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Award Number: DAMD17-03-1-0269

TITLE: Protein ISG15 Modification in the Development and the Treatment of Chronic Myeloid Leukemia

PRINCIPAL INVESTIGATOR: Dong-Er Zhang, Ph.D.

CONTRACTING ORGANIZATION: The Scripps Research Institute
La Jolla, CA 92037

REPORT DATE: June 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
30-06-2007	Final	1 JUN 2003 - 31 MAY 2007

4. TITLE AND SUBTITLE
Protein ISG15 Modification in the Development and the Treatment of Chronic Myeloid Leukemia

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0269

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dong-Er Zhang, Ph.D.

Email: dzhang@scripps.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Scripps Research Institute
La Jolla, CA 92037

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Interferons are useful drugs in treating chronic myeloid leukemia (CML). One of the cellular responses of interferon treatment is the activation of protein modification by ISG15. We have cloned a novel gene encoding a protease UBP43 that specifically removes ISG15 from ISG15 modified proteins. Furthermore, we have generated UBP43 knockout mice. UBP43 deficient hematopoietic cells have much higher levels of ISG15 modified proteins upon interferon stimulation and are hypersensitive to interferon treatment. This grant is to demonstrate that protein ISG15 modification is crucial for interferon function in CML treatment and to analyze the effect of UBP43 on CML development. In the past funding period, we have characterized BCR-ABL positive leukemia cell lines that have higher than normal or lower than normal levels of ISG15 conjugation. Furthermore, we have completed the studies on UBP43 knockout mice in the resistance to BCR/ABL induced CML development and demonstrated that interferon plays a critical role in the process. One important finding is that the effect of UBP43 in interferon signaling is independent of its function in protein ISGylation.

15. SUBJECT TERMS
knockout mice, cell proliferation, retroviral mediated gene expression, protein modification

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	81	19b. TELEPHONE NUMBER *(include area code)*

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Introduction:

Interferons are widely used in the treatment of cancers, especially chronic myeloid leukemia (CML). Although a recently developed new drug - imatinib mesylate (STI571) has shown tremendous success in treating CML, interferons will continually be a crucial player in CML treatment, especially to patients who have developed resistance to imatinib. One of the cellular responses of interferon treatment is the activation of protein modification by ISG15. We have cloned a novel gene encoding a protease UBP43 that specifically removes ISG15 from ISG15 modified proteins. Furthermore, we have generated UBP43 knockout mice. UBP43 deficient hematopoietic cells have much higher levels of ISG15 modified proteins upon interferon stimulation and are hypersensitive to interferon treatment. Most importantly, mice transplanted with wild type bone marrow cells with BCR-ABL expression rapidly develop a myeloproliferative disorder resembling human CML. In contrast, mice transplanted with BCR-ABL expressing UBP43 deficient bone marrow cells have not developed the CML-like disease. Therefore, we hypothesize that (1) Increase of protein ISG15 modification in response to interferon is critical to the efficacy of interferon and (2) inhibiting UBP43 to increase protein ISG15 modification will significantly increase the efficacy of interferon in the treatment of CML. This grant funding is to demonstrate that protein ISG15 modification is crucial for interferon function in CML treatment and to analyze the effect of UBP43 on CML development.

Body:

Task 1. To demonstrate that protein ISGylation is crucial for interferon function in CML treatment:

a. To generate UBE1L expressing K562 cells (Completed).
b. To study the effect of protein ISG15 modification on interferon responsiveness (Completed).
c. To analyze the correlation of protein ISGylation and interferon response in primary human CML samples (Initiated and then stopped based on newly obtained preliminary data in order to focus on more important study about the molecular mechanism of UBP43 in IFN signal transduction).

ISG15 is a small protein encoded by an interferon stimulated gene (ISG) (1-3). Its expression is highly induced upon interferon treatment. ISG15 is comprised of two domains, both of which have homology to ubiquitin (4). The N-terminal and C-terminal domains of ISG15 are 33% and 32% identical to ubiquitin, respectively. Upon interferon treatment, ISG15 can be detected in cells both in the free and conjugated form (5). In most cell types and tissues protein ISGylation is almost undetectable under normal conditions.

There is a series of distinct enzymes involved in the process of protein ubiquitination and deubiquitination, including ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), ubiquitin – protein ligase (E3), and the ubiquitin proteases (ubp) (6-8). In contrast, the enzymes involved in protein ISGylation have not been so well studied yet. A gene encoding a protein (UBE1L) homologous to the ubiquitin-activating enzyme E1 has been cloned during the analysis of chromosomal 3p21 deletions associated with small cell lung cancer (9). The chromosomal 3p21 deletion is also associated with non-small cell lung cancer and other solid tumors (10).

Furthermore, immunohistochemical analysis has revealed that UBE1L is expressed in normal lung cells, but not in 14 human lung cancer cell lines (11). These studies indicate that UBE1L may play an important role in the prevention of cancer development. A recent study reports that the influenza B virus protein, NS1B, blocks protein ISGylation via its direct interaction with ISG15. Further analysis by the same group indicates that UBE1L is an E1 for protein ISG15 modification (12). We and another group have recently identified Ubc8 as ISG15 E2 (13,14). Most recently, we have identified that estrogen responsive finger protein (EFP) can function as an E3 enzyme in ISGylation (15).

During the analysis of genes differentially expressed between wild type and leukemia fusion protein AML1-ETO knock-in mice, we have cloned a novel gene product and named it UBP43 (16,17). The predicted amino acid sequence indicates that UBP43 is a member of the UBP family of ubiquitin specific proteases. UBP43 contains the conserved domains, including the Cys and His domains, that are present in all UBP family members (6,7,18,19). In addition, it has little homology to other family members outside the two conserved regions. Our functional analysis of UBP43 demonstrated that it is an ISG15 specific protease (20). To understand the role of UBP43 and protein ISGylation, we generated UBP43 knockout mice. UBP43-/- cells have higher interferon induced protein ISGylation than UBP43+/+ and +/- cells. Furthermore, UBP43-/- cells are hypersensitive to interferon treatment. These findings lead to the hypothesis that inhibiting UBP43 enzyme activity during interferon cancer therapy may significantly enhance the efficacy of interferon. Furthermore, in contrast to the rapid development of a myeloid proliferation disorder with BCR-ABL expressing wild type bone marrow cells, UBP43-/- cells do not develop such a disease, indicating that UBP43 plays a crucial role in the regulation of myeloid cell proliferation during leukemogenesis.

It has been reported that the expression of interferon stimulated genes were increased in both interferon sensitive and resistant CML patients, indicating the major defect of interferon resistance is not at the level of interferon signaling and is at the level of post-translational modification (21). The K562 cell line is a hematological malignant BCR-ABL expressing cell line derived from a 53 year old female CML patient (22). Compared to many other cells, K562 cells are resistant to interferon induced suppression of cell proliferation (23). Since it has been reported that ISG15 activating enzyme UBE1L gene deletion is associated with small cell lung cancer development, we decided to study whether K562 cells lack UBE1L for ISG15 conjugation. After addition of a UBE1L protein expression construct into K562 cells by transient transfection, we can clearly detect ISGylated proteins upon interferon treatment (24). This result indicates that lack of protein ISGylation may contribute to resistance to interferon treatment in K562 cells. Therefore, to study the role of protein ISGylation in the interferon response, we decided to establish K562 cell lines expressing UBE1L and also to directly investigate the correlation between the interferon response and protein ISGylation in primary CML patient hematopoietic cells as stated in task I.

Since we were not successful in establishing UBE1L expressing K562 cell lines with multiple tries as described in the previous report, we have focused our effort in modulating protein ISG15 modification levels in KT-1 cells. KT-1 is another BCR-ABL positive leukemia cell line. In contrast to K562 cells, KT-1 cells are sensitive to interferon treatment and have a clear increase of ISGylation upon interferon treatment. Using short hairpin RNA (shRNA) for the RNA interference approach, we have generated KT-1 cell lines with control shRNA, UBE1L shRNA, and UBP43 shRNA via retroviral infection. Knockdown of UBP43 expression results in a large

increase in protein ISGylation upon IFN treatment and knockdown UBE1L expression lowers the amount of ISGylation in KT-1 cells (**Fig. 1**).

With these cell lines, we performed studies to examine cell proliferation. KT-1 cells with control shRNA, UBE1L shRNA, and UBP43 shRNA were cultured in the absence or presence of 1,000 unit/ml human interferon alpha (hIFNα) for 72 hours. The number of cells in the culture was checked daily. In the absence of hIFNα, we did not detect any dramatic change in cell proliferation upon the expression of the three different shRNAs. In contrast, with the hIFNα treatment, an obvious decrease in cell proliferation was observed in all three cell lines. By 72 hours of treatment, growth of cells with control and UBE1L shRNAs were reduced to 40% and 43% of untreated cells, respectively; growth of cells with UBP43 shRNA were reduced to 21% of untreated cells (**Fig. 2**). These results indicate that the absence of UBP43 has a more dramatic effect on the total number of cells upon IFN treatment. The alteration of protein ISGylation did not significantly change the growth rate. One possibility is that the total ISGylation level in parental KT-1 cells is not high enough to make a really clear comparison between control and UBE1L shRNA cells. Therefore, we also in the middle of creating UBP43/UBE1L double knockdown cells to study ISGylation effect.

The alteration of cell growth may be due to different apoptotic rates or different proliferation rates. To distinguish between these two possibilities, we performed both Annexin V/ 7-Aminoactinomycin D (7-AAD) double staining apoptosis assays and Propidium Iodide (PI) staining cell cycle assays. Relative to cells not treated with IFN, cells treated with 1,000 unit/ml hIFNα did not show any obvious change in apoptosis at 24 hours (**Fig. 3**). By 40 – 48 hours, we clearly detected the increase of apoptotic cells in all three shRNA expressing cells. Importantly, by 72 hours of hIFNα treatment, UBP43 shRNA expressing cells had about two-fold more apoptotic cells compared to control and UBE1L shRNA expressing cells. To analyze further the sensitivity of these cells to IFN, we treated these cells with 100 unit/ml hIFNα. The lower concentration of IFN reduced the severity of apoptosis in all three types of cells (**Fig. 4**). However, more obvious differences of IFN responses were detected in the three cell populations. More than four times more apoptotic cells were detected in cells expressing UBP43 shRNA relative to control and UBE1L shRNA expressing cells. This data is in agreement with our previous finding that UBP43 deficient cells are more sensitive to IFN induced apoptosis (24). The change of protein ISGylation between control and UBE1L shRNA expressing cells did not affect their IFN induced apoptosis.

IFN is also known to inhibit cell proliferation in some leukemia cell lines by a block at the G0/G1 phase of the cell cycle (25). We therefore also performed cell cycle analyses to check whether IFN stimulated KT-1 lines would show a block in a specific phase of the cell cycle. Cells were stained with propidium iodide and analyzed by flow cytometry. A minor increase in G0/G1 cells at 24 hours post treatment was observed (**Fig. 5**). The three types of cells showed a similar effect. This increase in G0/G1 cells is absent by 40 hours and cells treated with IFN for 48 hours actually showed a slightly lower percentage in the G0/G1 phase. These results indicate that IFN treatment for 48 hours does not inhibit cell cycling in KT-1 at any specific phase. At time periods greater than 48 hours, DNA from apoptotic cells became a significant interference and prevented good curve-fitting of the various cell cycle phases.

Considering both the apoptosis and cell cycle studies, the results indicate that knockdown of UBE1L by expressing UBE1L shRNA decreased protein ISG15 conjugation, but did not substantially affect cell cycle and apoptosis with IFN treatment. In contrast, knockdown of UBP43 by expressing UBP43 shRNA increased protein ISG15 modification and cell apoptosis, but did not block the cell cycle at a particular phase to decrease the number of cells with IFN

treatment. These results did not support that ISG15 modification played a role in modulating UBP43 related IFN sensitivity. Furthermore, as shown in the Appendix Publication #1, we also generated control and UBP43 shRNA expressing KT-1 cells and with KT-1 cells expressing wild type UBP43 or an enzymatically inactive mutant of UBP43. We then examined the role of UBP43 in down-regulation of IFN induced JAK-STAT signaling pathway by assessing JAK activation in human KT-1 cells (**Fig. 6**). The inhibition of endogenous UBP43 in KT-1 cells by specific siRNA significantly extended the phosphorylation of both JAK1 and TYK2 kinases (phosphorylation was still detectable at 4 hours of IFN stimulation versus 1 hour in control cells). Conversely, KT-1 cells constitutively expressing either wt or mutant form of Ubp43 exhibited reduced levels of JAK1 and TYK2 phosphorylation. Together, the results from these assays indicate that UBP43, but not its ISG15 deconjugating enzyme activity, plays a critical role in modulating IFN signal transduction.

Task 1c. To analyze the correlation of protein ISGylation and interferon response in primary human CML samples.

To evaluate the correlation of protein ISGylation and interferon response in primary human CML samples, during the first year of the funding time, we received frozen peripheral blood mononuclear cell samples from untreated CML patients at the diagnosis stage of the disease. The number of cells in each sample is about 0.5×10^7 cells per vial. In order to investigate the effect of interferon α on the colony growth of CML cells with the aim of then relating this to the degree of ISG15 conjugation detected in these cells following IFN treatment, the semi-solid methylcellulose culture technique was used to measure the CFU-GM formation. Cells were cultured in methylcellulose in the presence of 20 ng/ml each of recombinant human GM-CSF, G-CSF, and IL-3. As these were CML samples, each patient sample was plated at a range of cell numbers, from 5×10^3 cells to 2×10^5 cells and colony growth counted at days 7, 10, and 14. A colony was counted as 50 or more cells. Of the four samples plated, two showed no colony growth at all and in the third sample, colony growth was detected only in assays with the higher cell numbers of 1×10^5 and 2×10^5 cells plated, which gave 55 and 100 colonies, respectively. In the fourth CML sample colonies were detected at all cell numbers plated except with 5×10^3 cells, the lowest cell number plated (**Fig. 7**). Furthermore, the fourth CML sample was also set up at 1×10^5 cells/ml in liquid culture with the addition of growth factors and with and without human IFNα. Cell numbers were counted by Trypan blue exclusion every 24 hours for 144 hours (**Fig. 8**).

After setting up the conditions for further studies according to the original proposal, we obtained preliminary data as described in Task 1b. These data suggested that UBP43 itself, but not protein ISG15 modification, plays a more important role in regulating IFN signal transduction. Therefore, we decided to first focus on the studies about the molecular mechanism of how UBP43 affected IFN signal transduction. As shown in **figure 9**, using transient transfection, immunoprecipitation, and western blotting, we identified the direct interaction between UBP43 and IFN receptor subunit R2 (IFNAR2), but not another subunit R1 (IFNAR1) or the correlated type II IFN receptor subunit R1 (IFNGR1). In order to determine the functional consequences of UBP43-IFNAR2 interaction, we tested whether UBP43 competes with JAK1 to form a complex with IFNAR2 in order to block JAK1 activation. We co-expressed JAK1 and IFNAR2 in 293T cells in the absence or presence of increasing amounts of UBP43 and performed a set of reciprocal pull down assays either for JAK1 or IFNAR2. As shown in **figure 10A**, UBP43 was capable of interfering with JAK1-IFNAR2 complex formation in a dose-dependent manner. In

order to determine whether UBP43 confers this mode of regulation under physiological conditions, we used the RNAi approach followed by the assessment of interaction between endogenous IFNAR2 and JAK1. First we confirmed that endogenous UBP43 specifically interacts with endogenous IFNAR2 upon IFN stimulation in human KT-1 cells (**Fig. 10B**). Furthermore, knock down of UBP43 in KT-1 cells by UBP43-specific siRNA increased the association between endogenous IFNAR2 and JAK1 (**Fig. 10B**), indicating that the endogenous UBP43 is capable of competing with JAK1 for IFN receptor binding. These data provide the genetic evidence to support the hypothesis that Ubp43-mediated titration of JAKs away from the receptor inhibit downstream phosphorylation cascade events. With these data in hand, it seems not necessary to put too much additional effort in study whether the level of ISGylation in primary patient data is related to IFN response. We therefore focused on Task 2 studies with the UBE1L knockout mouse model.

Task 2. To analyze the effect of UBP43 on CML development.

a. To study protein ISG15 modification and UBP43 expression in leukemic cells upon interferon stimulation (Completed).
b. To investigate the role of UBP43 in CML development (Completed).
c. To study interferon sensitivity of UBP43+ and UBP43- cells in the presence or in the absence of BCR-ABL expression (Completed).

We have reported that UBP43 deficient cells are hypersensitive to interferon treatment (Appendix Publication #1). Our studies have shown that UBP43 deficient hematopoietic cells are resistant to the development of BCR-ABL induced CML development in the retrovirus mediated bone marrow transplantation model. We have completed the first part of the study and published the work in Blood (Appendix Publication #2). Mice transplanted with wild type bone marrow cells expressing BCR-ABL developed CML like disease within five weeks (**Fig. 11A**). When the mice become moribund, they generally have 10 to 100 times more total white blood cells in their peripheral blood (**Fig. 11B**) and show splenomegaly and hepatomegaly (**Fig. 12A**). UBP43 deficient bone marrow cells are more resistant to leukemia development (**Fig. 11A**). Furthermore, there are also much less neutrophil infiltrations in the liver and spleen when they eventually develop CML (**Fig. 12B**). Our analyses demonstrate that when mice developed the CML like disease, there is a 4-fold increase of type I interferon concentration in their sera (**Fig. 13A**). Furthermore, UBP43 expression is much higher in the spleen of CML mice compared to control spleen samples (**Fig. 13B**). We next generated UBP43 and type I interferon receptor R1 subunit (IFNAR1) double knockout mice by crossing UBP43 knockout mice with IFNAR1 knockout mice. IFNAR1 is critical for type I interferon signal transduction. Unlike the UBP43 knockout mice, the double knockout mice developed a CML like disease, upon BCR-ABL expression, with a time course similar to that of wild type mice, indicating type I IFN signal transduction is required for the delay of CML development of UBP43 deficient bone marrow cells (**Fig. 14**).

The dimerization of BCR-ABL through the BCR portion results in autophosphorylation and constitutive activation of the ABL kinase in BCR-ABL and causes aberrant activation of growth and survival signaling pathways in the cell. The high level of BCR-ABL expression is required for its oncogenic effect. Therefore, we also studied whether the level of UBP43 protein and protein ISGylation affect the amount of BCR-ABL protein and the activation

(phosphorylation) of BCR-ABL using KT-1 cells and KT-1 cells expressing different types of shRNA in the presence or the absence of IFN stimulation. Two independent pools of stably transfected cells were used in the analysis. To examine the amount of BCR-ABL in these cells, we used anti-ABL western blotting and total cell lysates prepared from these cells. To study the autophosphorylation (activation) of BCR-ABL, we performed immunoprecipitation with anti-ABL antibody and western blotted the immunoprecipitates with antibodies against phosphorylated tyrosine. Neither BCR-ABL expression nor its activation was affected by the level of UBP43 expression (UBP43 shRNA) or protein ISGylation (UBE1L shRNA) (**Fig. 15A**). Furthermore, IFN treatment also did not affect BCR-ABL expression or activation (**Fig. 15B**).

Originally, we hypothesized that the resistance of CML development of UBP43 deficient cells is due to the increased sensitivity of these cells to type I IFN by greatly enhanced protein ISGylation of these cells. However, as reported in the task I section, our studies revealed that the increased type I IFN sensitivity is mainly due to the absence of UBP43, but not due to the increased protein ISGylation. The remaining question is whether ISGylation affects CML development via a different pathway. Therefore, we also performed retrovirus mediated BCR-ABL expression and bone marrow transplantation assays using bone marrow cells from wild type and UBE1L deficient mice (26). The preliminary data with reasonable number of recipient mice in the transplantation experiments did not show significant enough contribution of UBE1L to CML development (**Fig. 16**). However, the question still remains whether UBE1L plays a critical role in responding to IFN treatment of CML mice.

Key Research Accomplishments:

- Generated and characterized UBE1L knockdown BCR-ABL+ KT-1 cells.
- Generated and characterized UBP43 knockdown BCR-ABL+ KT-1 cells.
- Defined the importance of UBP43 in type I interferon signal transduction and discovered that its function in interferon signaling is independent of its ISG15 deconjugating enzyme activity.
- Published one report in EMBO Journal on the above finding.
- Characterized the effect of UBP43 and protein ISGylation on BCR-ABL expression and activation.
- Published one report about the role of UBP43 in CML development in Blood.
- Initiate an animal model to examine the role of protein ISGylation in CML development.

Reportable Outcomes:

Two manuscripts published.

1) Malakhova OA, Kim KI, Luo JK, Zou W, Kumar KGS**,** Fuchs SY, Shuai K, Zhang D-E. UBP43 suppresses interferon signaling independent of its enzymatic activity towards ISG15, 2006, EMBO J, 25:2358-67.

2) Yan M, Luo JK, Ritchie KJ, Ren R, Zhang D-E. Ubp43 negatively regulates BCR-ABL leukemogenesis via the Type I interferon receptor signaling, 2007, Blood, 110:305-12.

Conclusions:

During the funding period, we characterized both UBE1L and UBP43 shRNA expressing KT-1 cells in the response to interferon treatment. The results indicate that protein ISGylation (UBE1L shRNA to knockdown ISGylation) did not affect BCR-ABL expression/activation or interferon signaling in KT-1 cells, but that the decreased expression of UBP43 (UBP43 shRNA) enhanced interferon induced cell apoptosis and did not show an effect on BCR-ABL expression/activation in KT1 cells. Furthermore, we demonstrated that the effect of UBP43 is independent of its ISG15 deconjugating enzyme activity. Secondly, we complete the study of CML development using bone marrow cells from wild type and UBP43 deficient mice and demonstrate the resistance of CML development in the absence of UBP43.

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Appendices:

Please see attached two published papers.

Figures and Figure Legends:

Please see next 16 pages.

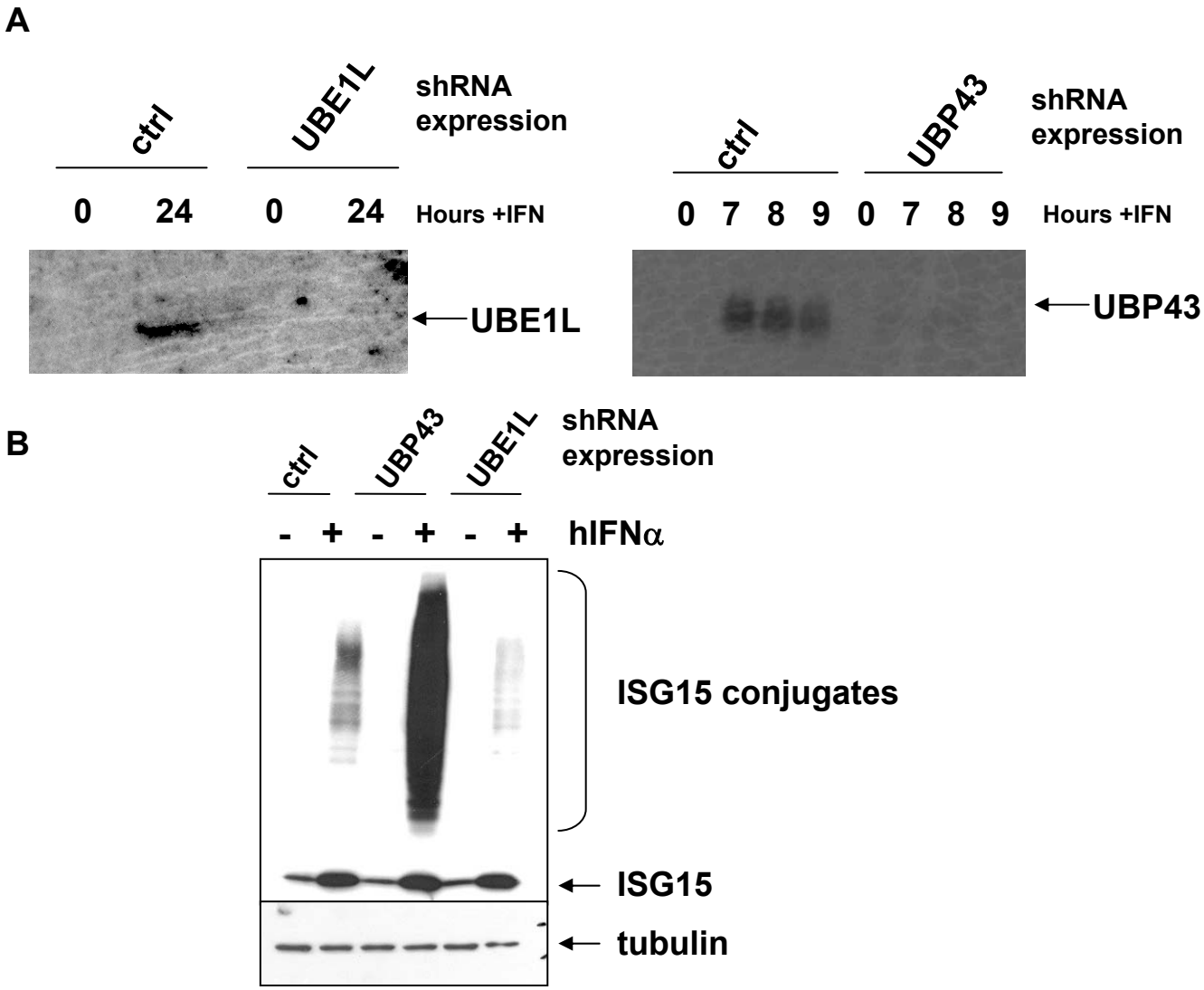

Figure 1. shRNA successfully knocks down the expression of UBP43 and UBE1L in KT-1. KT-1 cells were stably transduced with control (ctrl) shRNA or shRNA to either hUBP43 or hUBE1L and treated with 1,000 units/ml hIFNα for various time points. **A)** Left panel: Northern blot of KT-1 cells transduced with ctrl shRNA or UBE1L shRNA and probed for UBE1L expression. Right panel: KT-1 cells transduced with ctrl shRNA or UBP43 shRNA and probed for UBP43 expression. **B)** Cells were treated with (+) or without (-) 1,000 units/ml hIFNα for 48h. Knockdown of UBP43 and UBE1L were judged, respectively, by the increase and decrease of total ISGylation.

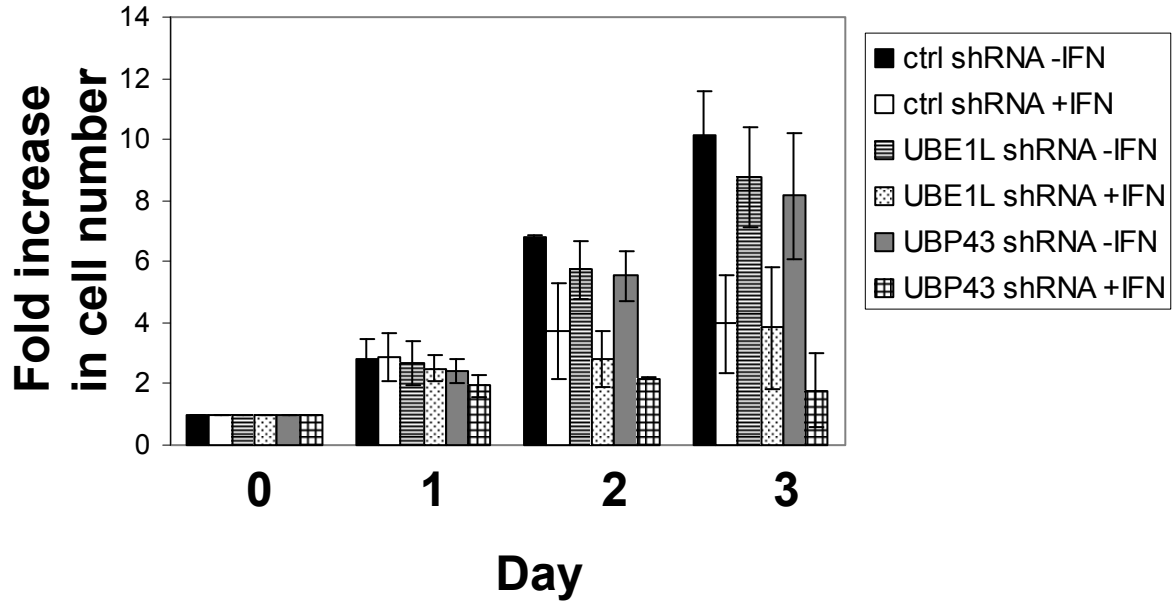

Figure 2. IFN induced growth suppression is unchanged in KT-1 cell lines expressing UBE1L shRNA but enhanced in UBP43 shRNA expressing cells. KT-1 cells stably transduced with the various shRNAs were treated with 1,000 units/ml hIFNα and their growth rate monitored over 72 hours. Cells were seeded at 1-2 x 105 cells/ml and the fold increase in cell number shown as the mean $\pm$ standard deviation (SD) of three separate experiments. UBP43 shRNA expressing cells consistently showed lower cell numbers than control shRNA or UBE1L shRNA expressing cells after 72 hours incubation with IFN.

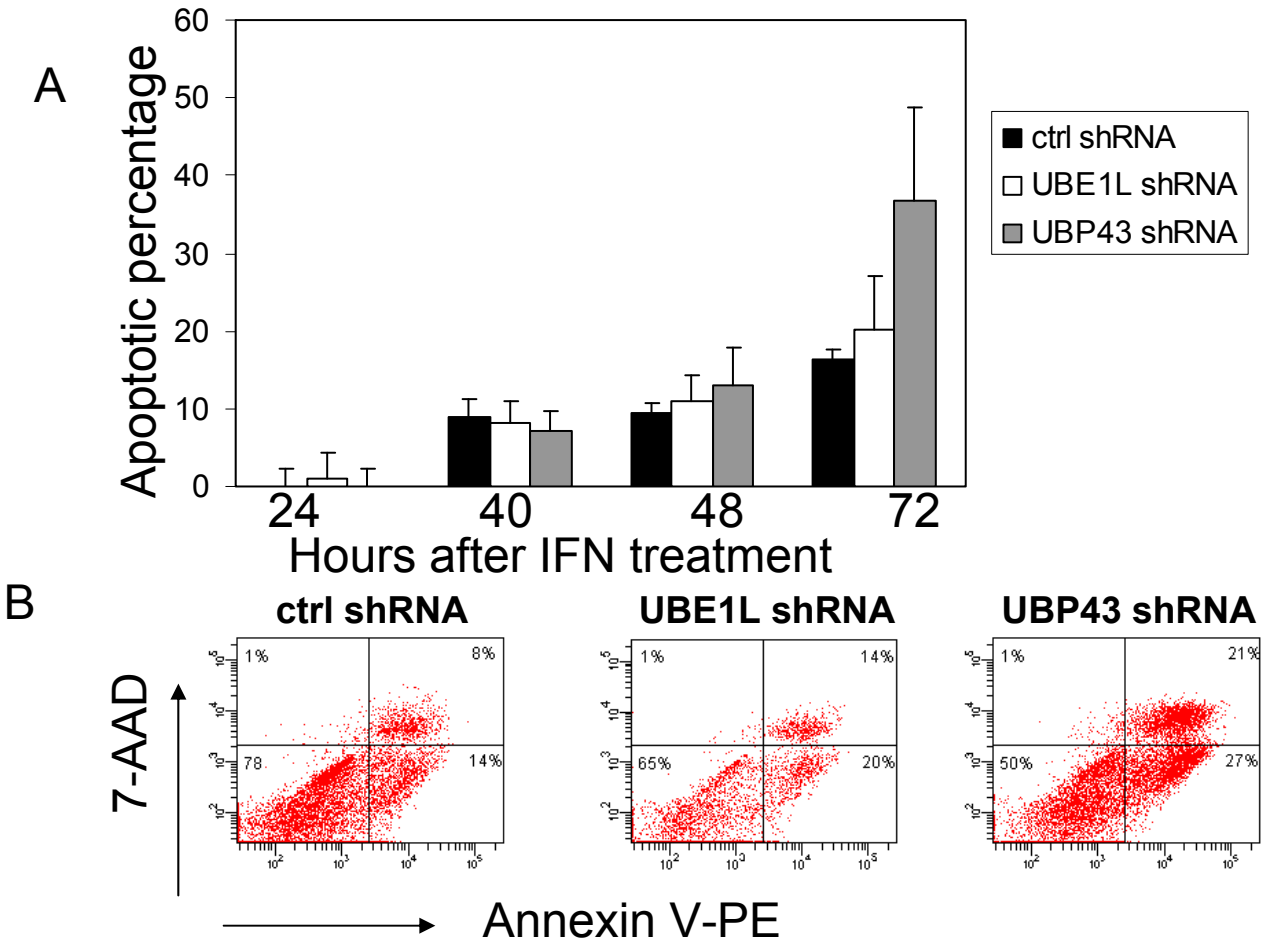

Figure 3. UBP43 shRNA expression, but not UBE1L shRNA expression, enhances IFN mediated cell death in KT-1. A) KT-1 cells stably transduced with the various shRNAs were treated with 1,000 units/ml hIFNα and the percentage increase in apoptotic cells (over control untreated cells) was determined by Annexin V/7-AAD staining at various time points. The apoptotic percentage represents the sum of early (Annexin V positive) and late apoptotic (Annexin V/7-AAD double positive) percentages. The results are the mean ± SD of three separate experiments. A comparison of the apoptotic percentage at 72h in control shRNA expressing cells to that of UBE1L shRNA and UBP43 shRNA expressing cells, yields *P* values of 0.40 and 0.05, respectively. **B)** Representative flow cytometry dot plot analysis of Annexin V/7-AAD stained cells. The percentages in the quadrants represent the percentage of total cell numbers in the respective quadrants. At 72 hours with IFN, UBP43 shRNA KT-1 show increased populations of both early and late apoptotic cells, as compared to control shRNA or UBE1L shRNA expressing cells.

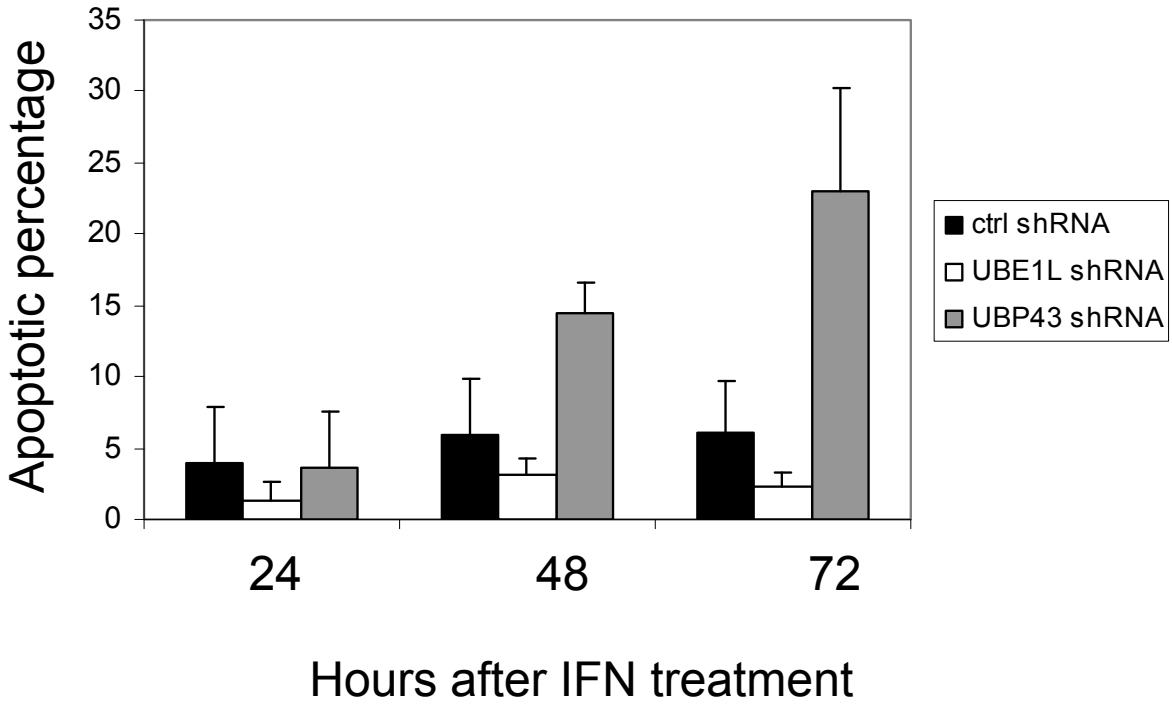

Figure 4. UBP43 shRNA expression, but not UBE1L shRNA expression, sensitizes KT-1 cells to induction of apoptosis by low doses of IFN. KT-1 cells stably transduced with the various shRNAs were treated with 100 units/ml hIFNα and the percentage increase in apoptotic cells (over control untreated cells) was determined by Annexin V/7-AAD staining at various time points. The apoptotic percentage represents the sum of early (Annexin V positive) and late apoptotic (Annexin V/7-AAD double positive) percentages. The results are the mean $\pm$ SD of three separate experiments. A comparison of the apoptotic percentage at 72h in control shRNA expressing cells to that of UBE1L shRNA and UBP43 shRNA expressing cells, yields *P* values of 0.10 and 0.02, respectively.

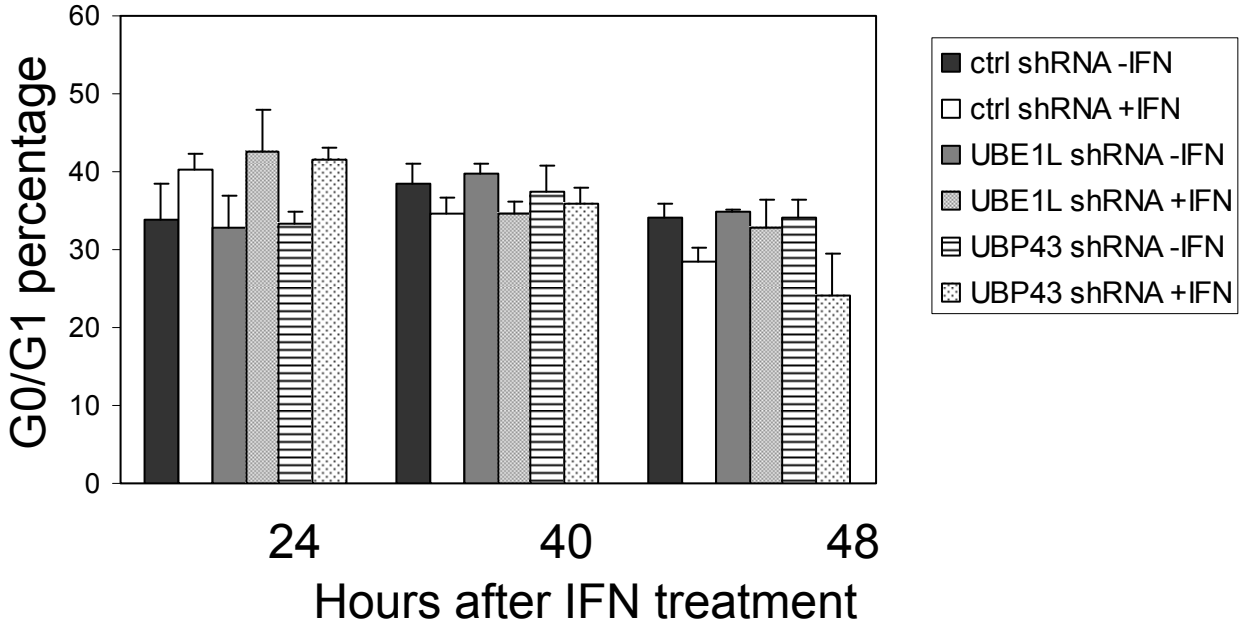

Figure 5. Expression of shRNA to UBE1L or UBP43 does not affect the percentage of cells in G0/G1 after IFN treatment. Cells were treated with 1,000 units/ml hIFNα and stained with 50 μg/ml propidium iodide. The percentage of G0/G1 cells represents the percentage of cells with 2N DNA content. Results are the mean $\pm$ SD of three separate experiments. All cell lines show a minor increase in G0/G1 cells at 24 hours after IFN treatment, although this difference decreases with increasing time. The amount of apoptotic cells at time points greater than 48 hours interfered with curve fitting of the histograms and was therefore not analyzed.

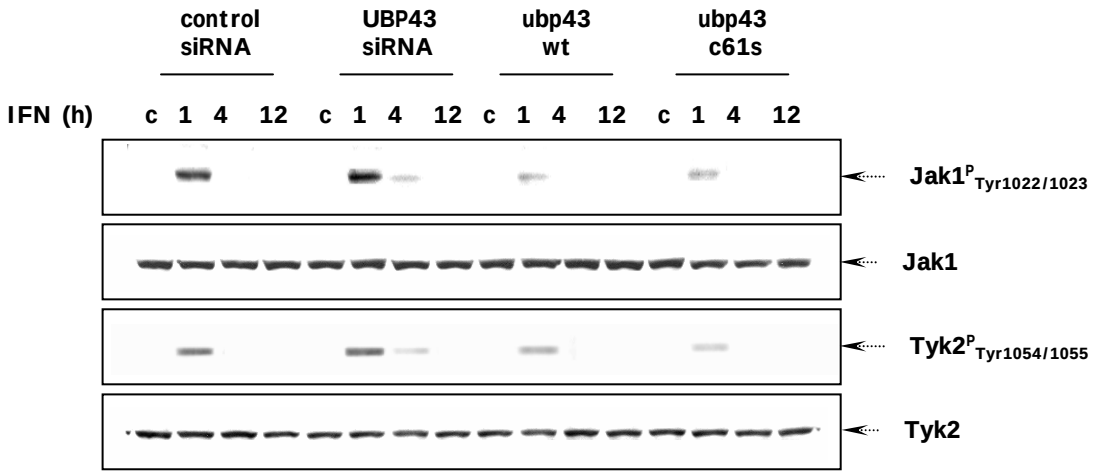

Figure 5. Ubp43 inhibits the activation of JAK-kinases. KT-1 cells stably expressing control siRNA, human UBP43-specific siRNA, plasmids encoding wt Ubp43 or Ubp43C61S mutant protein were stimulated with hIFN-α (1,000 U/ml) for the indicated periods of time. Whole cell lysates were subjected to immunoblotting with anti-phosphospecific JAK1 and TYK2 antibodies. Blots were stripped and re-probed with anti-JAK1 and TYK2 antibodies respectively to assure equal protein loading.

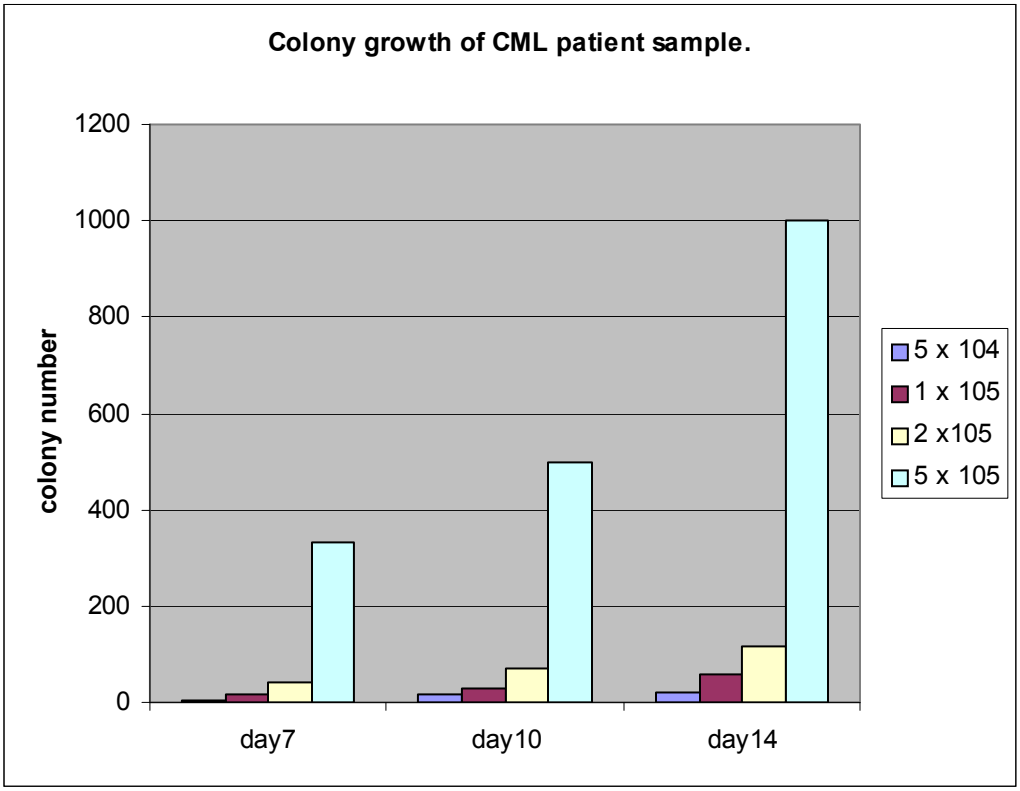

Figure 7. The clonogenic growth of human CML patient cells. Cells from a CML patient were cultured in methycellulose in the presence of 20 ng/ml each of recombinant human GM-CSF, G-CSF, and IL-3. the number of cells used in each plate of culture and the time of colonies counted were indicated in the figure.

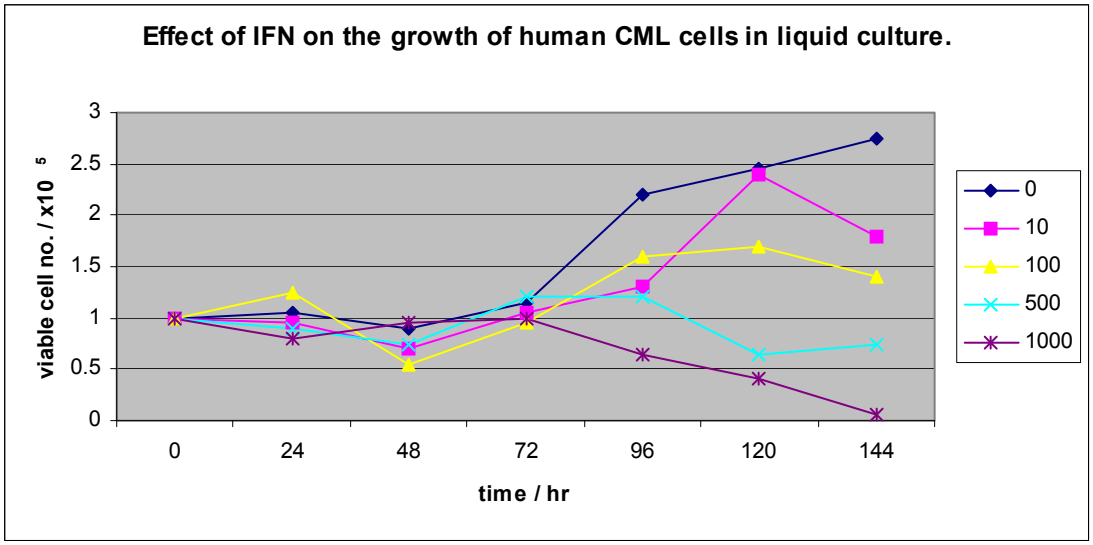

Figure 8. The effect of interferon on the growth of CML cells in liquid culture. Cells collected from a CML patient were cultured in RPMI medium containing 20 ng/ml each of recombinant human GM-CSF, G-CSF, and IL-3 in the presence of various concentrations of interferon α as indicated in the figure. The number of cells were counted daily with Trypan blue exclusion to study the effect of interferon on cell proliferation.

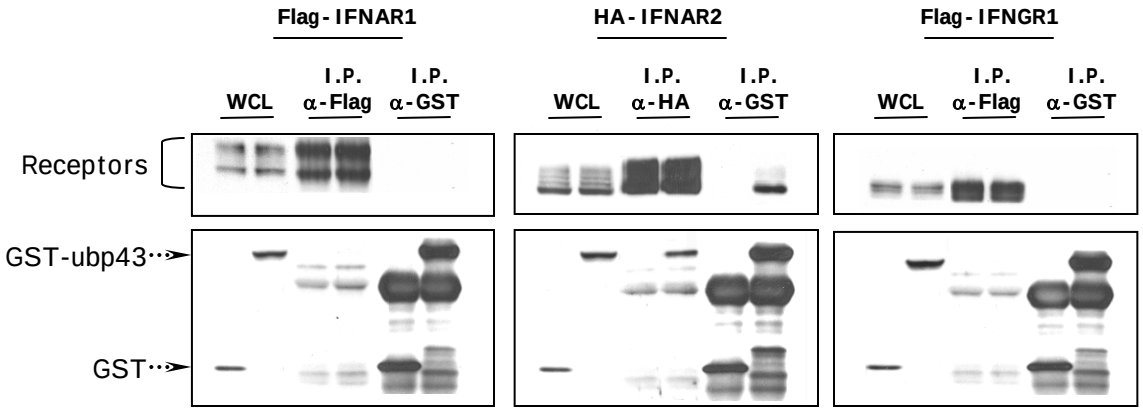

Figure 9. Ubp43 interacts with IFNAR2 receptor subunit. 293T cells were transiently transfected with Flag-IFNAR1, HA-IFNAR2, or Flag-IFNGR1 and either GST control or GST-Ubp43. Reciprocal immunoprecipitations (I.P.) were performed using anti-Flag/HA or anti-GST antibodies. Whole cell lysates (WCL) or immunoprecipitated complexes were subjected to immunoblotting with anti-HA antibodies (*top middle panel*), anti-Flag (*top left & right panels*) or anti-GST (*bottom panel*) antibodies, respectively.

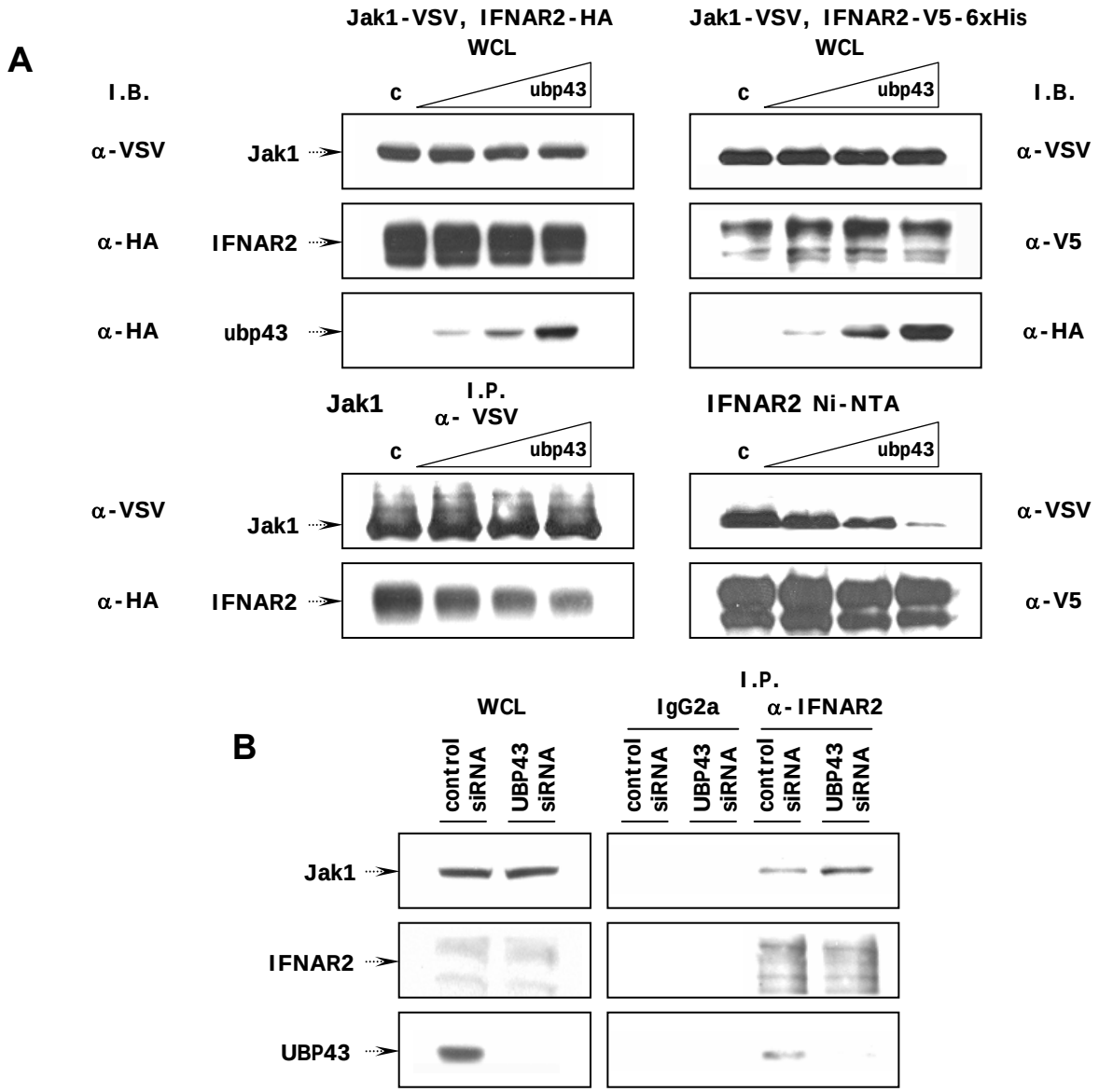

Figure 10. Ubp43 competes with JAK1 for receptor binding. (A) 293T cells were transiently co-transfected with JAK1-VSV and IFNAR2-HA (*left*) or JAK1-VSV and IFNAR2-V5-6xHis (*right*) in the absence or presence of increasing concentration of HA-Ubp43 followed by immunoprecipitation using antibodies against VSV-tagged JAK1 (*left, bottom panels*) or Ni-NTA purification of 6xHis-tagged IFNAR2 (*right, bottom panels*). Whole cell lysate (*top panels*) or immunoprecipitated complexes (*bottom panels*) were subjected to immunoblotting with antibodies indicated in the figure. (B) Protein extracts from stable KT-1 transfectants expressing either control siRNA or UBP43 specific siRNA and treated with hIFN-α for 4 hours were used for the immunoprecipitations with control IgG2a antibodies or with anti-IFNAR2 antibodies. Whole cell lysates and immunoprecipitates were subjected to immunoblotting with anti-JAK1 (*top panel*), anti-IFNAR2 (*middle panel*), and anti-Ubp43 antibodies (*bottom panel*).

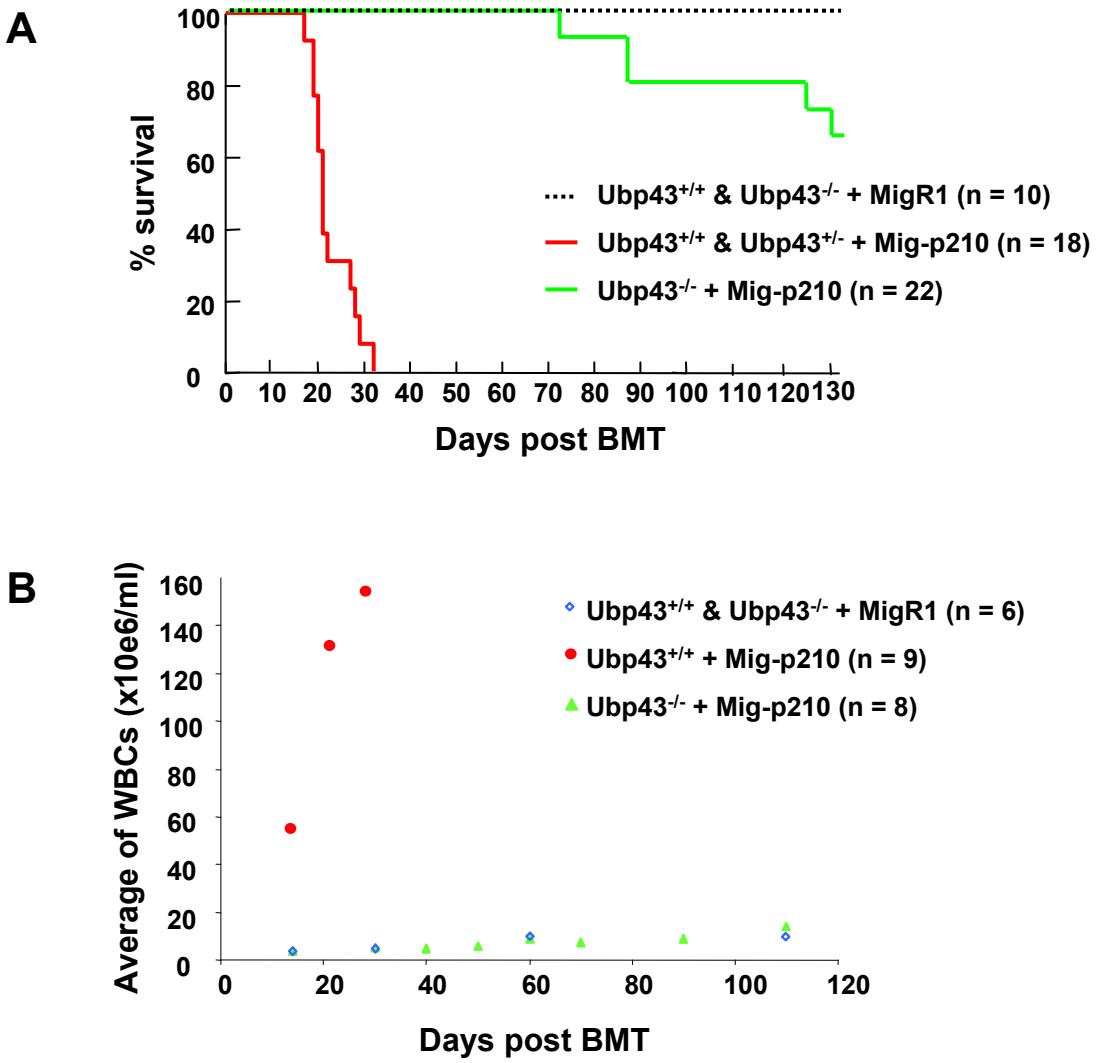

Figure 1. Significant delay of CML development with Ubp43 deficient bone marrow cells in BCR-ABL retroviral transduction/transplantation assay. (A) Kaplan-Meier survival curve of mice transplanted with BCR-ABL expressing retrovirus Mig-p210 or retroviral vector control MigR1 transduced Ubp43+/+, Ubp43+/-, and Ubp43-/- bone marrow cells. The result is summarized from three separate sets of transplantation experiments. (B) Average total white blood cell (WBC) counts of MigR1 or Mig-p210 transduced Ubp43+/+ and Ubp43-/- bone marrow cell recipients.

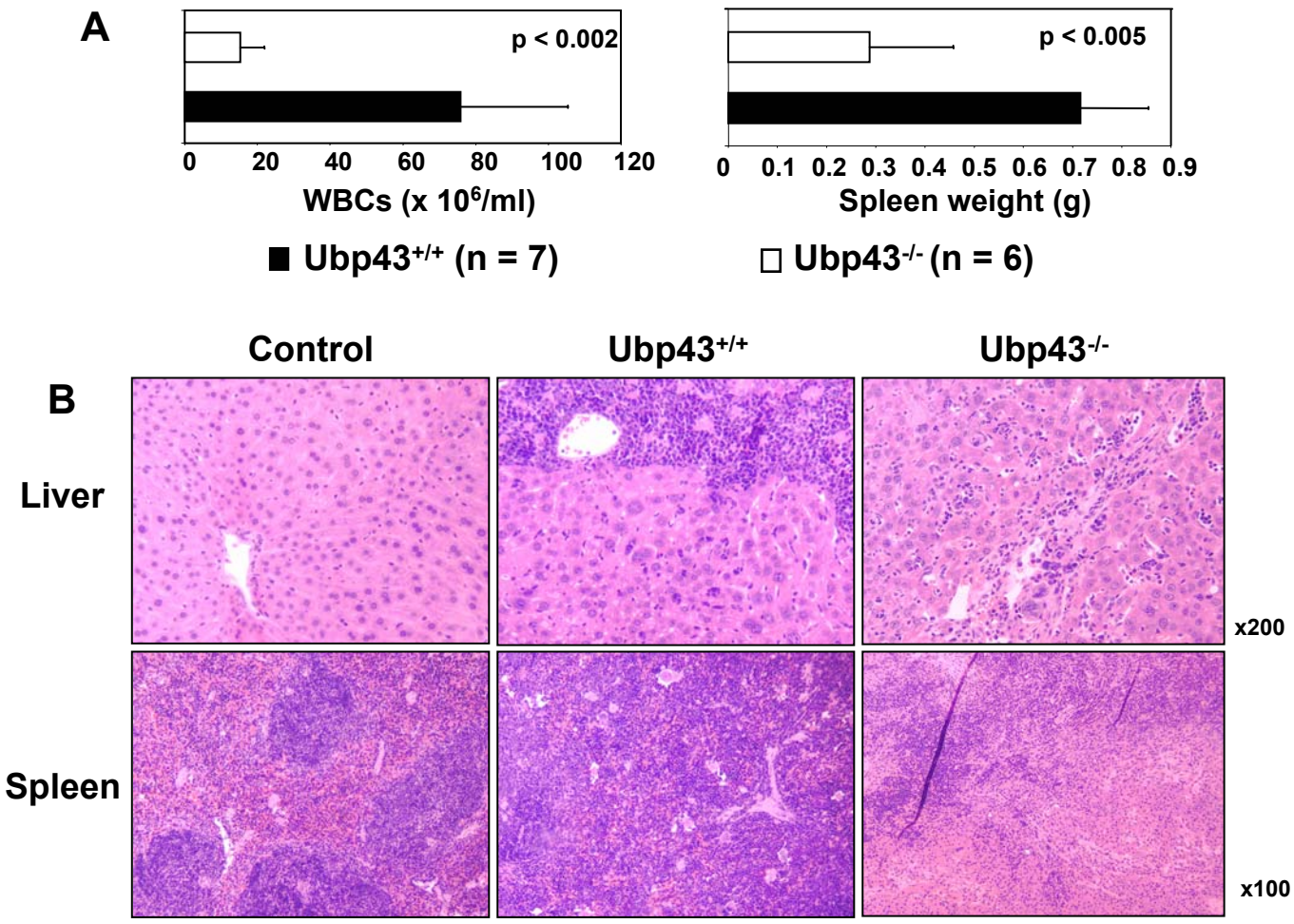

Figure 12. Pathological analysis of disease mice. (A) The average WBC counts and spleen weight of transplant recipients at moribund. The error bars represent the standard deviation. (B) Histological analysis of spleens and livers of representative control and experimental mice transplanted with Mig-p210 transduced Ubp43+/+ and Ubp43-/- bone marrow cells. The tissue sections were stained by hematoxilin and eosin.

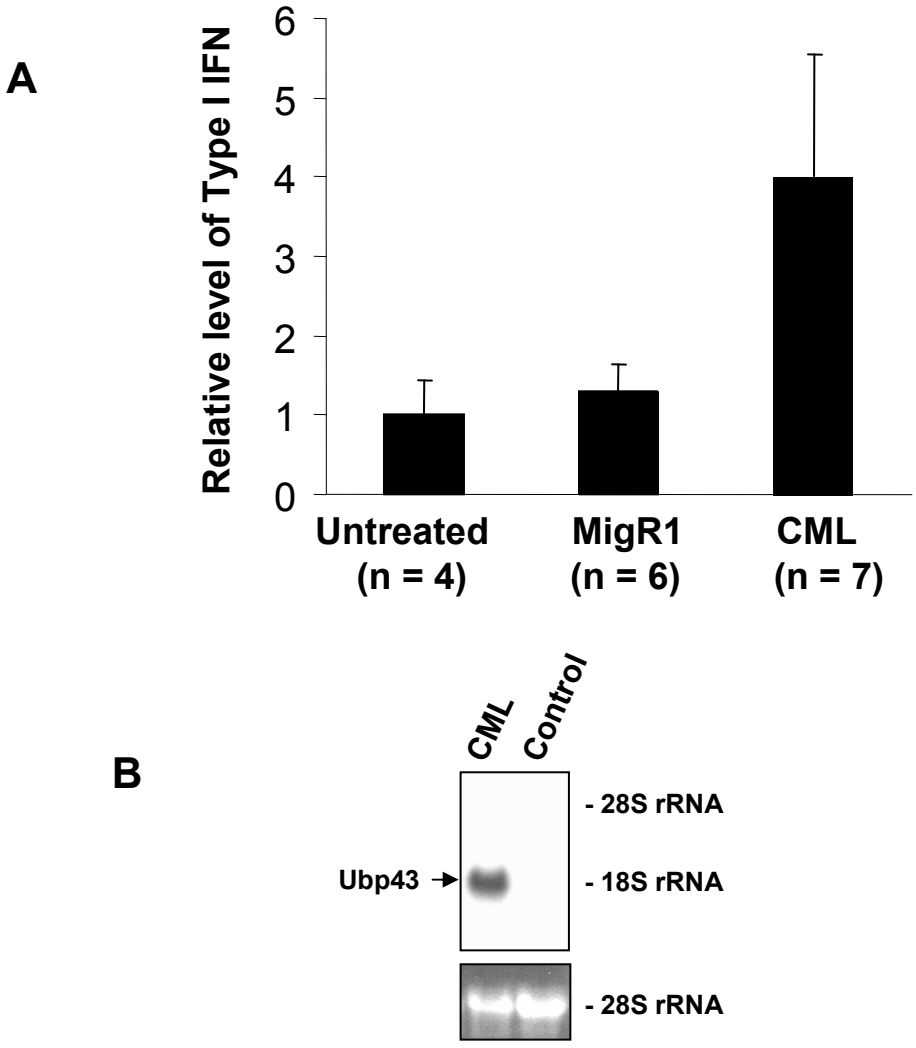

Figure 13. A. Elevated Type I IFN level is detected in the serum of mice with CML like disease. Serum was collected from control, MigR1 transplanted, and BCR-ABL induced CML mice.. The concentration of Type I IFN in these sera was measured as described in Materials and Methods. The relative concentrations of IFN in these sera are presented. **B. Ubp43 is clearly detectable in the spleen of mice which develop the CML-like disease.** RNA was prepared from the spleen of a mouse transplanted with MigR1 transduced Ubp43+/+ bone marrow cells (control) and the spleen of a mouse with CML-like disease after transplantation with Mig-p210 infected wild type bone marrow cells (CML). Northern blot was performed with 32P-labeled Ubp43 cDNA. Ethidium bromide stained 28S rRNA is shown for relative RNA loading.

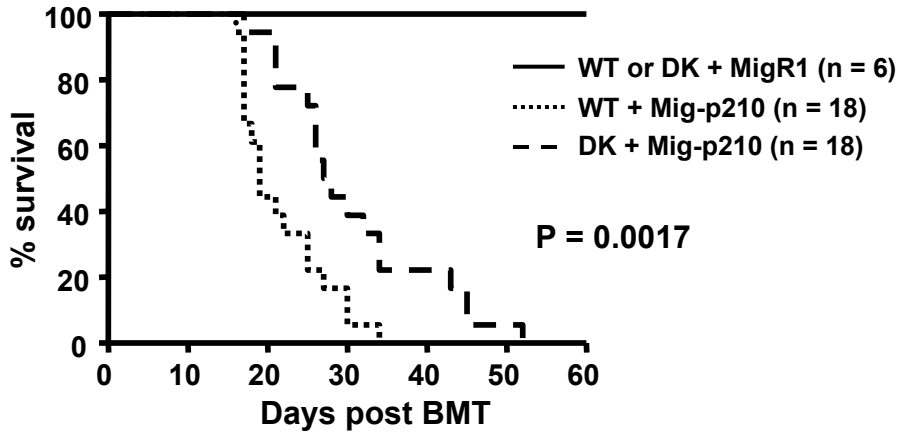

Figure 14. IFNα/β signaling plays a critical role in resistance to leukemia development in Ubp43 deficient cells. Kaplan-Meier survival curve of mice transplanted with MigR1 or Mig-p210 transduced wild type bone marrow cells (WT) or Ubp43 and IFNα/β receptor subunit R1 (Ifnar1) double deficient bone marrow cells (DK). The result is summarized from two separate sets of transplantation experiments.

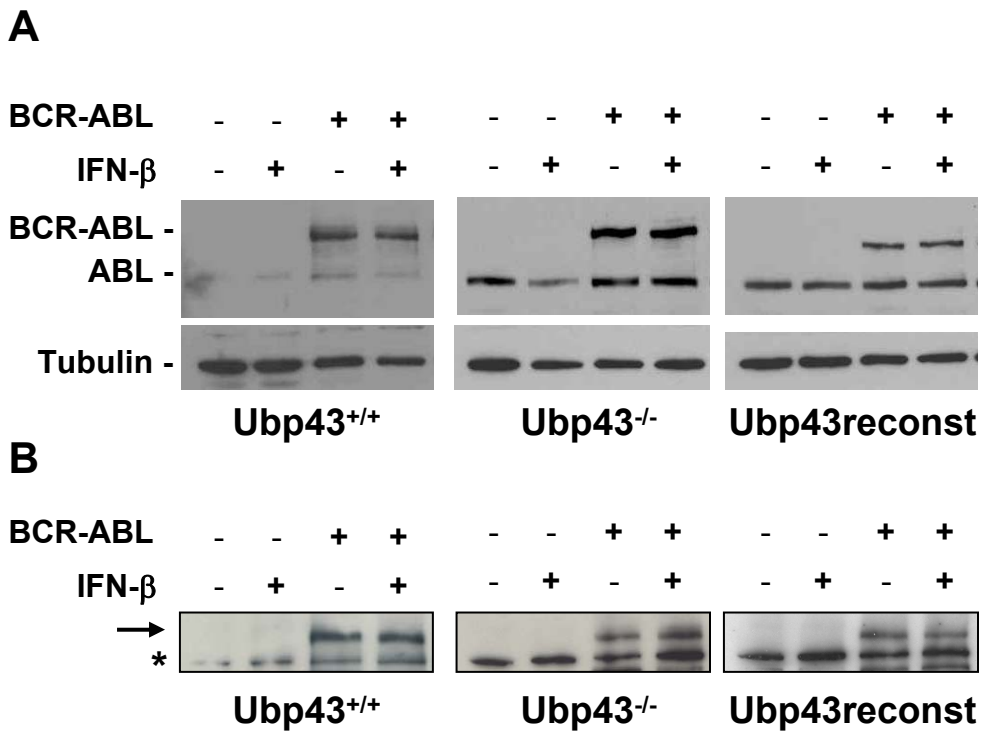

Figure 15. The expression and phosphorylation of BCR-ABL are not affected by the presence of Ubp43 or IFN-β. Protein extracts were prepared from parental Ubp43+/+, Ubp43-/-, and Ubp43 reconstituted Ubp43-/- (Ubp43reconst) MEFs and BCR-ABL expressing MEFs. (A) Ten μg of protein from each sample was used to western blot sequentially with α-ABL and α-tubulin antibodies. (B) The same protein samples were used to western blot with the antibody against phosphorylated tyrosine. BCR-ABL transduced lines showed a high molecular weight band (indicated by the arrow, > 175 kDa) corresponding to phosphorylated BCR-ABL, which is reduced upon STI571 treatment (data not shown). A constitutively phosphorylated protein (indicated by *) served as a loading control.

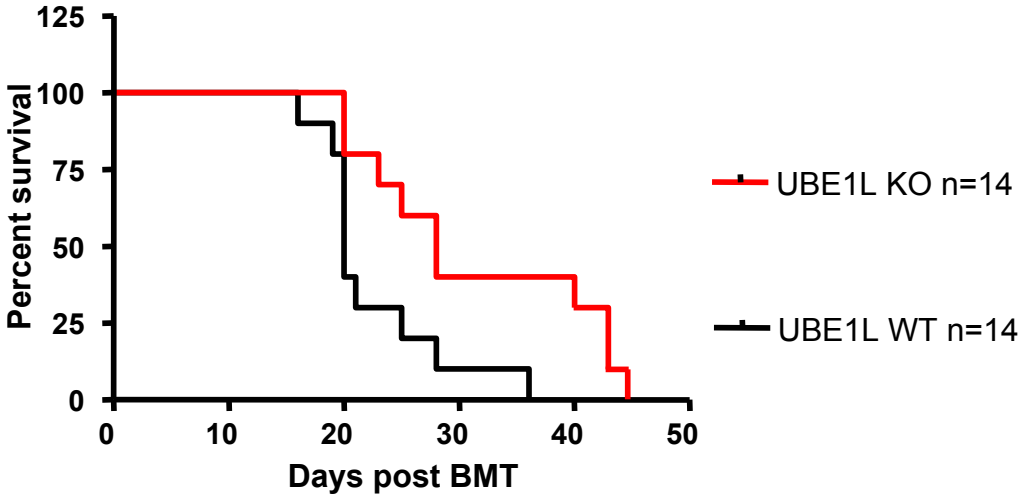

Figure 16. Kaplan-Meier survival curve of mice transplanted with UBE1L WT and KO bone marrow expressing BCR-ABL. Bone marrow cells from 5-FU treated Ube1L+/+ and Ube1L-/- mice were transduced with Mig-p210 and transplanted into lethally irradiated C57 recipient mice. Recipients of Ube1L-/- bone marrow were found to have a slight delay in the development of BCR-ABL induced leukemia.

The EMBO Journal (2006) 25, 2358–2367 | © 2006 European Molecular Biology Organization | All Rights Reserved 0261-4189/06
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THE EMBO JOURNAL

UBP43 is a novel regulator of interferon signaling independent of its ISG15 isopeptidase activity

Oxana A Malakhova[1], Keun Il Kim[1,4], Jiann-Kae Luo[1], Weiguo Zou[1], KG Suresh Kumar[2], Serge Y Fuchs[2], Ke Shuai[3] and Dong-Er Zhang[1,*]

[1]Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, USA; [2]Department of Animal Biology, University of Pennsylvania School of Veterinary Medicine, Philadelphia, PA, USA and [3]Division of Hematology/Oncology, School of Medicine, University of California at Los Angeles, Los Angeles, CA, USA

Interferons (IFNs) regulate diverse cellular functions through activation of the Janus kinase–signal transducer and activator of transcription (JAK–STAT) pathway. Lack of Ubp43, an IFN-inducible ISG15 deconjugating enzyme, leads to IFN hypersensitivity in *ubp43*−/− mice, suggesting an important function of Ubp43 in downregulation of IFN responses. Here, we show that Ubp43 negatively regulates IFN signaling independent of its isopeptidase activity towards ISG15. Ubp43 functions specifically for type I IFN signaling by downregulating the JAK–STAT pathway at the level of the IFN receptor. Using molecular, biochemical, and genetic approaches, we demonstrate that Ubp43 specifically binds to the IFNAR2 receptor subunit and inhibits the activity of receptor-associated JAK1 by blocking the interaction between JAK and the IFN receptor. These data implicate Ubp43 as a novel *in vivo* inhibitor of signal transduction pathways that are specifically triggered by type I IFN.

The EMBO Journal (2006) **25,** 2358–2367. doi:10.1038/sj.emboj.7601149; Published online 18 May 2006
Subject Categories: signal transduction; immunology
Keywords: interferon (IFN); ISG15; JAK; STAT; UBP43 (USP18)

Introduction

Interferons (IFNs) are secreted pleiotropic cytokines that regulate diverse biological functions, including induction of the antiviral response, inhibition of cell proliferation, and immunomodulatory activities (Platanias and Fish, 1999; Biron, 2001; Sen, 2001; Chawla-Sarkar *et al*, 2003; Pestka *et al*, 2004). Type I IFNs signal by binding to a cognate receptor at the cell surface followed by activation of the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway (Darnell *et al*, 1994; Levy and Darnell, 2002; Shuai and Liu, 2003). JAK1 and TYK2, two members of the JAK family of nonreceptor tyrosine kinases, constitutively associate with the IFNAR2 and IFNAR1 subunits of the type I IFN receptor, respectively. Binding of IFN to the receptor triggers heterodimerization of the receptor chains, juxtaposing JAKs and initiating the phosphorylation cascade (Aaronson and Horvath, 2002). Activated JAK1 phosphorylates TYK2 and they further phosphorylate the cytoplasmic tails of IFNAR proteins, creating a docking site for STAT1/STAT2 binding and their subsequent phosphorylation. Once phosphorylated, STAT1/2 heterodimers disengage from the receptor, form a complex with p48 (IRF9), translocate to the nucleus and induce gene expression through binding to the ìnterferon stimulated response element (ISRE) within the promoters of interferon stimulated genes (ISGs) (Fu, 1995; Larner and Reich, 1996; Decker *et al*, 2002).

Stringent mechanisms of signal attenuation are essential for ensuring an appropriate and controlled cellular response. Several mechanisms of negative regulation have been implicated in the termination of IFN signaling (Yamada *et al*, 2003), including IFN receptor ubiquitination by $SCF^{\beta\text{-TrCP/HOS}}$ E3 ubiquitin ligase and degradation by the lysosomal pathway, dephosphorylation of JAKs and the receptor by SHP-1 and SHP-2, dephosphorylation of STATs by TC45 and PTP1B, inhibition of STAT1 DNA binding by the family of PIAS proteins, and inhibition of JAK kinase activity and their subsequent degradation by SOCS proteins (Greenhalgh and Hilton, 2000; Yasukawa *et al*, 2000; Kumar *et al*, 2003; Shuai and Liu, 2005).

Along with other ISGs, IFN-α/β stimulation leads to upregulation of ISG15, a ubiquitin-like protein (Ubl) that conjugates to a number of cellular substrates (Narasimhan *et al*, 1995, 1996; Ritchie and Zhang, 2004). The conjugation involves an enzymatic cascade that includes an E1 activating enzyme (Ube1L) (Yuan and Krug, 2001), an E2 conjugating enzyme (Ubc8) (Kim *et al*, 2004; Zhao *et al*, 2004), and most likely some E3 ligases (Dao and Zhang, 2005; Zou and Zhang, 2006). The conjugation process is also reversible and controlled by an IFN-inducible cysteine protease of the ubiquitin-specific protease (USP) family of enzymes—Ubp43 (USP18) (Malakhov *et al*, 2002, 2003). Recently, we found that Ubp43 negatively regulates JAK–STAT signaling and may therefore represent a novel type of inhibitor in the IFN pathway (Malakhov *et al*, 2002, 2003). *Ubp43*-deficient cells exhibit high levels of ISG15 modified proteins (Ritchie *et al*, 2002). Furthermore, they are hypersensitive to type I IFN and undergo apoptosis upon IFN stimulation. Lack of Ubp43 results in enhanced and prolonged STAT1 phosphorylation, DNA binding, and increased induction of hundreds of ISGs as confirmed by gene expression microarray data (Malakhova *et al*, 2003; data in preparation for publication). As the consequence, loss of Ubp43 in mice results in greater resistance to the cytopathic effects caused by a number of viruses including lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), and Sindbis virus (SNV) (Ritchie

*Corresponding author. Department of Molecular and Experimental Medicine, The Scripps Research Institute, MEM-L51, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA. Tel.: +1 858 784 9558; Fax: +1 858 784 9593; E-mail: dzhang@scripps.edu
[4]Present address: Department of Biological Science, Sookmyung Women's University, Seoul 140-742, Korea

Received: 12 December 2005; accepted: 21 April 2006; published online: 18 May 2006

et al, 2004). Although Ubp43 apparently inhibits JAK–STAT signaling, the molecular mechanisms of such inhibition are yet to be determined. As isopeptidase activity toward removal of ISG15 from its substrate is the only currently known function of Ubp43 (Malakhov *et al*, 2002), it would be plausible that the negative regulation of IFN signaling by Ubp43 is mediated by de-conjugation of ISG15. However, ablation of ISG15 or Ube1L in mice does not reverse the phenotype of the Ubp43 knockout (Knobeloch *et al*, 2005; Osiak *et al*, 2005; Kim *et al*, 2006).

In this report, we investigated the functional mechanism of Ubp43 action within the IFN signaling pathway and showed that Ubp43 negatively regulates JAK–STAT signaling independently of its isopeptidase activity. Ubp43 action is strictly specific to type I IFN responses and achieved through a direct interaction between Ubp43 and the IFNAR2 subunit of the receptor. Binding of exogenous and endogenous Ubp43 to IFNAR2 *in vivo* interferes with the JAK-receptor interaction and leads to inhibition of the downstream phosphorylation cascade and other signaling events. These data point to a mechanistically novel nonenzymatic function of Ubp43 as a specific *in vivo* inhibitor of cellular responses to type I IFN.

Results

Neither ISG15 conjugation nor Ubp43 isopeptidase activity is required for the negative regulation of IFN signaling by Ubp43

Genetic ablation of Ubp43 in mice causes hypersensitivity to IFN and subsequent hyperactivation of ISGs, suggesting that the Ubp43 protein is a negative regulator of the JAK–STAT signaling pathway (Malakhova *et al*, 2003). The only reported function of Ubp43 is its enzymatic activity against ISG15 conjugates. Indeed, *ubp43*-deficient mice show high levels of ISG15-conjugated proteins in most tissues in response to IFN treatment. However, it remains to be determined whether sustained ISG15 conjugation may lead to IFN hypersensitivity in *ubp43*-null mice or if such hypersensitivity might be mediated by other mechanisms that are independent of Ubp43 enzymatic activity. To address both possibilities, we performed a set of experiments to separate the impact of the ISG15 conjugation process from any ISG15-independent function of Ubp43. For this purpose, we generated MEFs that included parental *ubp43*+/+ and *ubp43*−/− MEFs or stably transfected pools of *ubp43*−/− MEFs expressing Ube1L siRNA, as well as either wild-type Ubp43(wt) or an active site cysteine mutant (c61s) of Ubp43, Ubp43^{C61S}, that is no longer enzymatically active. First, we confirmed that reconstitution of *ubp43*−/− MEFs with Ube1L siRNA noticeably decreased the degree of ISG15 conjugation, bringing ISGylation to a level comparable with the *ubp43*+/+ cells upon IFN-β stimulation (Figure 1A, top panel). Reconstitution of *ubp43*−/− MEFs with constitutively expressed ubp43 (Figure 1A, second panel) resulted in a lower level of ISGylation than that of *ubp43*+/+ (Figure 1A, top panel). Unexpectedly, expression of the enzymatically inactive mutant of Ubp43 in *ubp43*−/− MEFs also resulted in lower levels of ISG15 conjugates although the ratio between conjugated and free ISG15 was higher in these cells than in cells expressing wt Ubp43. Given that both wt and mutant forms of Ubp43 were expressed at similar levels (Figure 1A, top two panels), it is likely that the decrease levels of ISG15 conjugation in cells transduced with catalytically inactive Ubp43 reflects an impaired IFN induced expression of free ISG15 and enzymes responsible for ISG15 conjugation and, therefore, might be indicative of inhibition of the JAK–STAT pathway in an isopeptidase-independent manner.

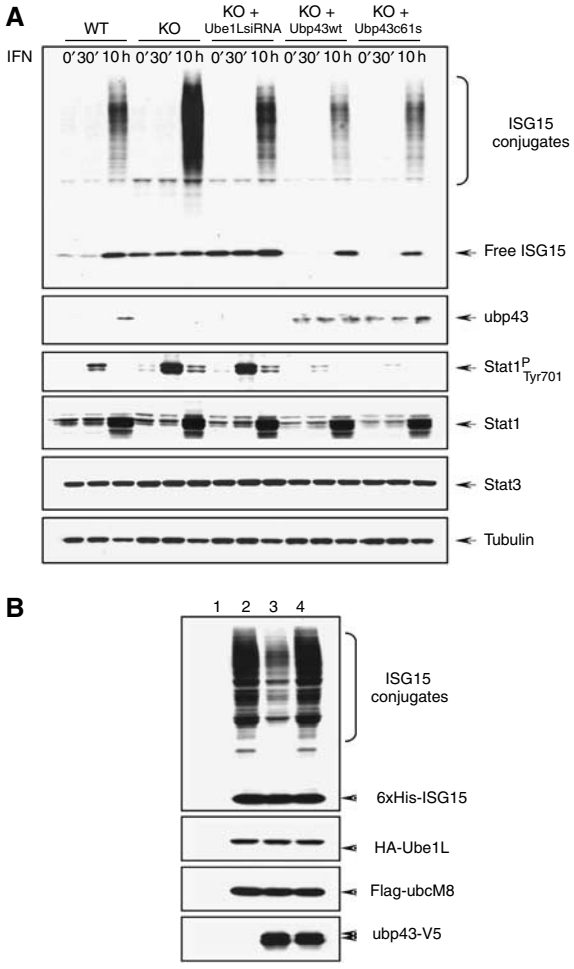

Figure 1 Negative regulation of IFN signaling by Ubp43 is independent of its enzymatic activity. (**A**) Total protein extracts were prepared from *ubp43*+/+ and *ubp43*−/− MEFs or *ubp43*−/− MEFs stably expressing Ube1L siRNA, wt murine Ubp43, or Ubp43^{C61S} after mIFN-β (1000 U/ml) treatment for 0′, 30′, and 10 h and analyzed by Western blotting with the respective antibodies. (**B**) 293T cells were transiently transfected with vector-control (lane 1) or plasmids containing 6xHis-ISG15, HA-Ube1L, and Flag-Ubc8 in the absence (lane 2) or the presence of wt (lane 3) or mutant (c61s) Ubp43-V5 (lane 4). The level of ISG15 conjugation was determined by Western blotting with anti-mISG15 antibodies. Blots were stripped and reprobed with anti-HA, anti-Flag, or anti-V5 antibodies to ensure equal levels of protein expression.

Indeed, reconstitution of *ubp43*−/− cells with either wt or mutant Ubp43 resulted in a significant inhibition of STAT1 tyrosine phosphorylation on the Tyr701 residue to a level similar to *ubp43*+/+ cells (Figure 1A, third panel), suggesting that catalytic activity of Ubp43 toward ISG15 conjugates is not required for this inhibition. This conclusion was further corroborated by data showing that downregulation of the total level of ISG15 conjugates in *ubp43*-deficient cells by inhibiting Ube1L function with specific siRNA did not affect the level of STAT1 phosphorylation compared to the parental *ubp43*−/− MEFs (Figure 1A). STAT3 and tubulin blots were

performed to ensure equal protein loading in this experiment (Figure 1A, fifth and sixth panels).

We also confirmed that Ubp43^{C61S} is enzymatically inactive *in vivo*. ISG15 conjugation can be artificially induced in 293T cells by overexpression of ISG15 and enzymes involved in its conjugation (Ube1L (E1), and Ubc8 (E2)) in the absence of IFN (Figure 1B, lane 2) (Kim *et al*, 2004; Zhao *et al*, 2004). When wt Ubp43 was co-expressed in 293T cells with this artificial ISG15-conjugation system, it significantly lowered the amount of ISG15 conjugates *in vivo* (Figure 1B, lane 3). In contrast, co-expression of Ubp43^{C61S} did not have any effect on the level of ISG15 conjugation (Figure 1B, lane 4). These data confirmed that the active site cysteine 61 to serine substitution completely abolished the enzymatic activity of Ubp43 towards ISG15.

To determine whether Ubp43 can function as a negative regulator of JAK–STAT signaling independent of the level of ISG15 conjugation at the physiological level, we performed a VSV protection assay (Wong *et al*, 2001) using MEF pools that included parental *ubp43+/+* and *ubp43−/−* MEFs, as well as *ubp43−/−* MEFs expressing Ube1L siRNA, wt Ubp43, or Ubp43^{C61S}. Consistent with previous observations (Ritchie *et al*, 2004), MEF cells deficient for Ubp43 resisted significantly higher VSV titers as compared to *ubp43+/+* cells (Figure 2) (10^5 versus 10^4 PFUs in untreated control and $>10^{10}$ versus 10^8 PFUs in IFN-β treated samples). Reconstitution of *ubp43−/−* cells with either wt or active site mutated Ubp43 reduced the resistance level back to that of *ubp43+/+* cells. However, downregulation of ISG15 conjugation by knocking down Ube1L did not significantly affect cellular resistance to VSV in *ubp43−/−* cells. These results, together with similar activities exhibited by wt Ubp43 and Ubp43^{C61S} in this assay, indicate that the lack of Ubp43 is important for the IFN-induced resistance of these cells to viral infections whereas ISG15 conjugation mediated by Ube1L is not essential.

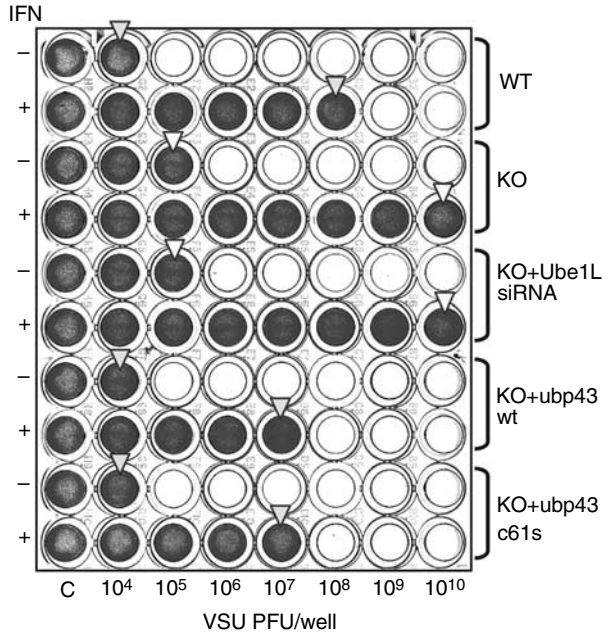

Figure 2 VSV protection assay. *ubp43+/+*, *ubp43−/−*, *ubp43−/−* MEFs with stable expression of Ube1L siRNA, wt Ubp43, or Ubp43^{C61S} were left untreated or treated with 1000 U/ml of mIFN-β for 24 h, followed by mock or VSV infection ranging from 10^4 to 10^{10} PFU/well for additional 24 h. Cell viability was assessed by crystal violet staining. The arrows indicate the level of protection.

Ectopic expression of wt or inactive mutant of Ubp43 blocks STAT1 phosphorylation and IFN-mediated gene induction

To determine the effect of forced Ubp43 expression on JAK–STAT signaling, we also generated stably transfected human U3A cells (STAT1-deficient cells, which were selected for this study based on their low basal level of endogenous UBP43) and KT-1 cell pools with different levels of Ubp43 expression. U3A cells constitutively expressing wt Ubp43 or Ubp43^{C61S} (Malakhov *et al*, 2002) were transiently transfected with STAT1 in combination with an ISRE-driven promoter-luciferase reporter. As shown in Figure 3A, left panel, the expression of either the enzyme active or inactive form of Ubp43 decreased the induction of STAT1 phosphorylation in response to IFN-α in U3A cells. In accordance with this observation, the level of IFN-specific promoter activation was found to be significantly lower in cells expressing wt or mutant Ubp43 (Figure 3A, right panel). Similar data were also obtained from KT-1 cells that stably expressed the wt or mutant Ubp43 (data not shown). Both cell lines showed a substantially reduced IFN response as judged by the level of STAT1 phosphorylation. In contrast, the knockdown of Ubp43 by specific siRNA in KT-1 cells extended the duration of IFN signaling (Figure 3B) mimicking the IFN hypersensitive phenotype observed in *ubp43*-knockout mouse cells (Figure 1A; Malakhova *et al*, 2003). These results provide additional genetic evidence in support of our conclusion that Ubp43 negatively regulates JAK–STAT signaling and subsequent activation of ISGs in response to IFN in both murine and human cells in a manner that is independent of Ubp43 enzyme activity toward ISG15 conjugates.

Cell surface expression and rate of degradation of IFN receptor are not altered in Ubp43 deficient cells

Given that de-conjugation of ISG15 does not play a role in the inhibition of JAK–STAT signaling by Ubp43, it is plausible that expression of Ubp43 may either affect IFN receptor downregulation or be involved in the activation/deactivation of JAK. We next tested whether Ubp43 affects ligand-induced downregulation of IFN receptor, which plays a primary role in restricting the extent and duration of IFN signaling. Treatment of cells with IFN was shown to trigger rapid IFNAR1 ubiquitination (Kumar *et al*, 2003) and degradation (Constantinescu *et al*, 1994); furthermore, stable IFNAR1 mutants that bear deletions in their C-terminus mediate enhanced responses to IFN-α (Gibbs *et al*, 1996; Basu *et al*, 1998).

We first investigated whether Ubp43 could affect the level of the endogenous IFNAR1 and IFNAR2 subunits of the receptor at the cell surface. Since antibodies against extracellular domains of IFN receptor subunits are only available for human proteins, we performed flow cytometry analysis of the cell surface level of both IFNAR1 and IFNAR2 using human KT-1 cells expressing either control siRNA or Ubp43-specific siRNA. Despite highly efficient UBP43 knockdown (Figure 4A), no apparent differences in steady state or IFN-induced surface levels of either IFN receptor subunit

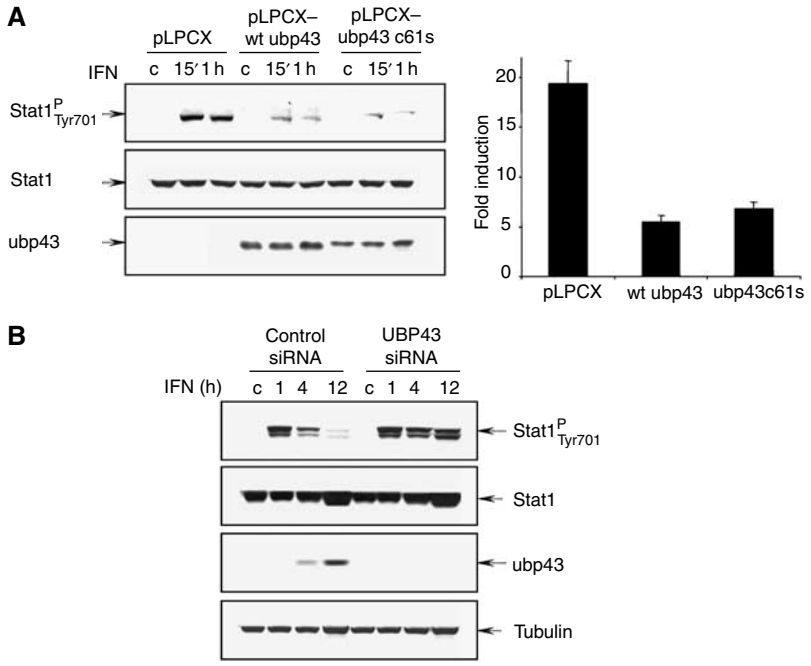

Figure 3 Ectopic expression of Ubp43 blocks STAT1 phosphorylation and IFN-mediated gene induction. (**A**) STAT1-deficient U3A stable cell lines expressing vector-control, wt mUbp43, or mUbp43^{C61S} were transiently co-transfected by STAT1 and ISRE-driven luciferase reporter plasmid. At 24 h post-transfection, cells were either left untreated or treated with hIFN-α for 15′, 1 h, and 16 h. Level of STAT1 phosphorylation and expression was assessed by Western blotting with the respective antibodies (left). Luciferase activities were measured, normalized, and presented as fold increase of relative luciferase activity in IFN treated cells (at 16 h point) over the untreated controls (right). The error bars indicate the s.d. of the mean. (**B**) KT-1 cells were stably transfected with control siRNA, hUBP43-specific siRNA. After 1 week of drug selection, cells were either left untreated or treated with hIFN-α for 1, 4 or 12 h respectively. STAT1 phosphorylation and expression was assessed by Western blotting with the respective antibodies. Specific inhibition of endogenous hUBP43 by siRNA in the respective stable lines was confirmed by Western blotting with anti-hUBP43-specific antibodies.

were detected in UBP43-defficient KT-1 cells as compared with control cells (Figure 4A), suggesting that Ubp43 is not involved in regulating the cellular surface level of IFN receptors.

We then analyzed the rate of receptor ubiquitination and degradation in MEF cells that express VSV-tagged murine IFNAR1 (as analysis of endogenous murine IFNAR1 is hindered due to the unavailability of appropriate mIFNAR1-specific antibodies). Neither the half-life of mIFNAR1-VSV (treated with IFN-β and cycloheximide, Figure 4B) nor ubiquitination of this receptor (data not shown) differed between *ubp43−/−* and *ubp43+/+* cells. These results indicate that Ubp43 is not involved in the control of either IFNAR1 ubiquitination or IFNAR1 proteolysis.

Ubp43 inhibits the phosphorylation of JAK

The magnitude and duration of IFN signaling is negatively controlled by phosphatases at different levels such as dephosphorylation of STATs, JAKs, and the receptor. On the other hand, given that JAK activation is the initial event in activation of the JAK–STAT signaling pathway, a sustained activation of JAKs could also positively affect components of this pathway. No difference in dephosphorylation regulation was detected between wt and *ubp43−/−* cells (data not shown). Therefore, we examined the phosphorylation rate of JAK1 in the presence of IFN and the phosphatase inhibitor sodium orthovanadate. Upon this treatment, we observed that re-expression of Ubp43 in *ubp43*-deficient MEF cells decreases the extent of JAK1 phosphorylation (Figure 5A). An increase in the magnitude and duration of IFN-induced JAK1 phosphorylation was also observed in the bone marrow cells derived from Ubp43 knockout mice as compared with cells from wt animals (Figure 5B). We further examined the role of Ubp43 in downregulation of the JAK–STAT signaling pathway by assessing JAK activation in human KT-1 cells (Figure 5C). The inhibition of endogenous UBP43 in KT-1 cells by specific siRNA significantly extended the phosphorylation of both JAK1 and TYK2 kinases upon IFN-α stimulation (phosphorylation was still detectable at 4 h of IFN stimulation versus 1 h in control cells). Conversely, KT-1 cells constitutively expressing either the wt or mutant form of Ubp43 exhibited reduced levels of JAK1 and TYK2 phosphorylation. Together, the results suggest that Ubp43 inhibits the tyrosine kinase activity of these JAKs in a manner that does not rely on the protease activity of Ubp43.

Ubp43 attenuates IFN signaling through the specific interaction with IFNAR2 subunit of the receptor

Ubp43 deficient cells are hypersensitive to type I IFN, but not to type II IFN (Malakhov *et al*, 2002, 2003). In line with these findings, we did not observe any effects of Ubp43 knockdown in human cells on the activation of the JAK–STAT pathway in response to IFNγ, IL-6, or IL-12 (data not shown). Furthermore, forced expression of Ubp43 did not affect the autophosphorylation of either Jak1 or Tyk2 induced by overexpression of the respective kinases in 293T cells in the absence of IFN stimulation (data not shown). The ability of Ubp43 to attenuate the activity of JAK in a type I IFN dependent manner suggested that it may be associated with the type I IFN specific component of the IFN-receptor com-

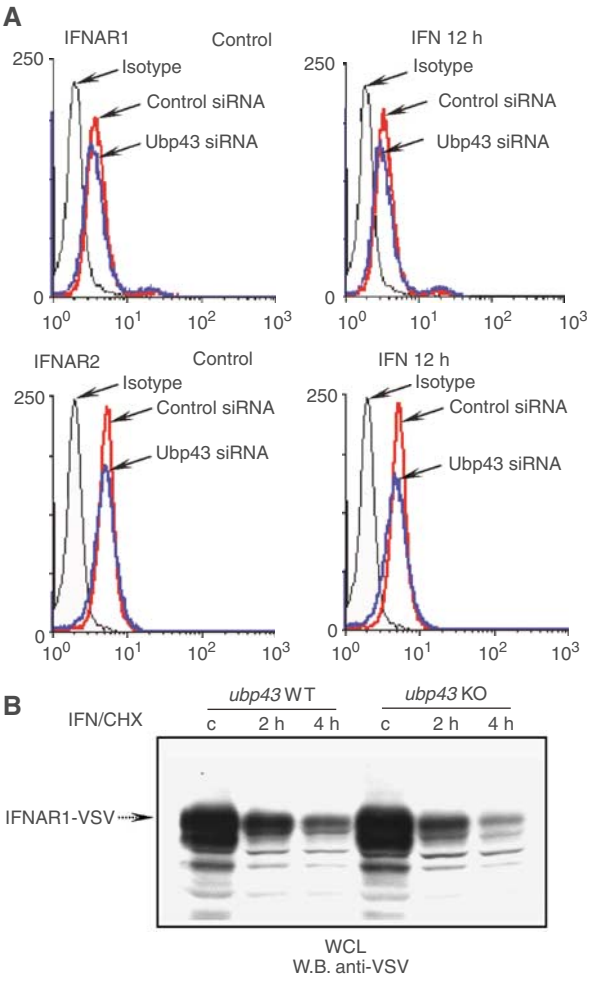

Figure 4 IFN receptor cell surface expression and degradation are not altered in Ubp43 deficient cells. (**A**) The steady-state or IFN-induced level of the endogenous IFNAR1 (upper left panels) and IFNAR2 (lower left panels) chains was determined by flow cytometry analysis of KT-1 cells, expressing control or UBP43-siRNA by using polyclonal anti-IFNAR1 and monoclonal MMHAR-2 antibodies, respectively. Graphs, corresponding to the isotype-matched control or IFNAR-specific staining in KT-1 control and UBP43-siRNA cells are marked by arrows. (**B**) Protein extracts were prepared from *ubp43*+/+ and *ubp43*−/− MEFs with stable expression of VSV-tagged mIFNAR1 that were either left untreated or treated with IFN and cycloheximide (CHX, to inhibit the protein synthesis) for 2 and 4 h respectively. The rate of IFNAR1 degradation was assessed by Western blotting.

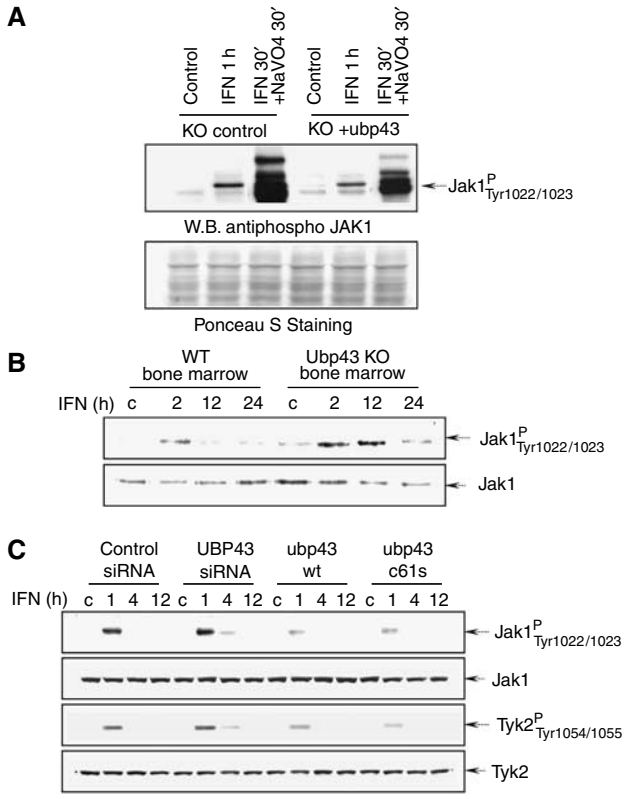

Figure 5 Ubp43 inhibits the activation of JAK-kinases. (**A**) Whole-cell lysates (WCL) were prepared from *ubp43*−/− MEFs or *ubp43*−/− MEFs reconstituted with wt mUbp43 after treatment with mIFN-β alone or mIFN-β + sodium orthovanadate for the indicated period of time. Level of JAK1 phosphorylation was determined by Western blotting using phospho-specific antibodies against JAK1 (pYpY1022/1023) (upper panel). After SDS–PAGE membrane was stained with Ponceau S solution to assure equal protein loading (lower panel). (**B**) Bone marrow cells were prepared from *ubp43*+/+ or *ubp43*−/− mice and incubated with 100 U/ml of mIFN-β for various periods of time as indicated in the figure. JAK1 was immunoprecipitated from WCL and subjected to an *in vitro* kinase assay, followed by immunoblotting with anti-phosphospecific JAK1 antibodies. Blots were stripped and re-probed with anti-JAK1 antibodies. (**C**) KT-1 cells stably expressing control siRNA, human UBP43-specific siRNA, plasmids encoding wt Ubp43 or $Ubp43^{C61S}$ mutant protein were stimulated with hIFN-α (1000 U/ml) for the indicated periods of time. WCL were subjected to immunoblotting with antiphosphospecific JAK1 and TYK2 antibodies. Blots were stripped and re-probed with anti-JAK1 and TYK2 antibodies respectively to assure equal protein loading.

plex and that both IFNAR1/IFNAR2 receptor chains represent potential targets for Ubp43 action. To determine whether these proteins are in a complex with Ubp43, we performed reciprocal co-precipitation assays for the assessment of Ubp43-receptor interactions. Specific binding of the IFNAR2 subunit of the type I IFN receptor to Ubp43 was detected (Figure 6A, middle). Neither IFNAR1 (Figure 6A, left) nor IFNGR1 (type II IFN) receptor subunits (Figure 6A, right) were able to interact with Ubp43. Furthermore, both wt Ubp43 and $Ubp43^{C61S}$ were found to bind to IFNAR2 to a similar extent (Supplementary Figure S1).

To further delineate the region of Ubp43 responsible for the interaction with IFNAR2, a panel of GST fusion proteins encoding portions of Ubp43 was used for binding studies (Figure 6B, top). Following co-expression/co-immunoprecipitation assays, we found that the Ubp43–IFNAR2 interaction was mediated by the C-terminus of Ubp43, since the deletion of the N-terminal portion of Ubp43 up to amino acid (a.a.) 217 did not affect its interaction with IFNAR2 (Figure 6B, middle panel). Meanwhile, GST-fusions expressing various fragments encompassing the N-terminal two-thirds of Ubp43 protein (up to a.a. 243) or GST alone were not able to bind IFNAR2 (Figure 6B, middle panel, lanes 1, 4 and 5). Further truncation from the amino terminus revealed that a 56-a.a. fragment (312–368) of Ubp43 binds to IFNAR2, as efficiently as the full-length protein (Figure 6B, bottom panel, lane 5). From these data we concluded that the very C-terminus of Ubp43 provides the main interaction motif for the association with IFNAR2 and residues in the region of a.a.312–368 might be critical for the interaction.

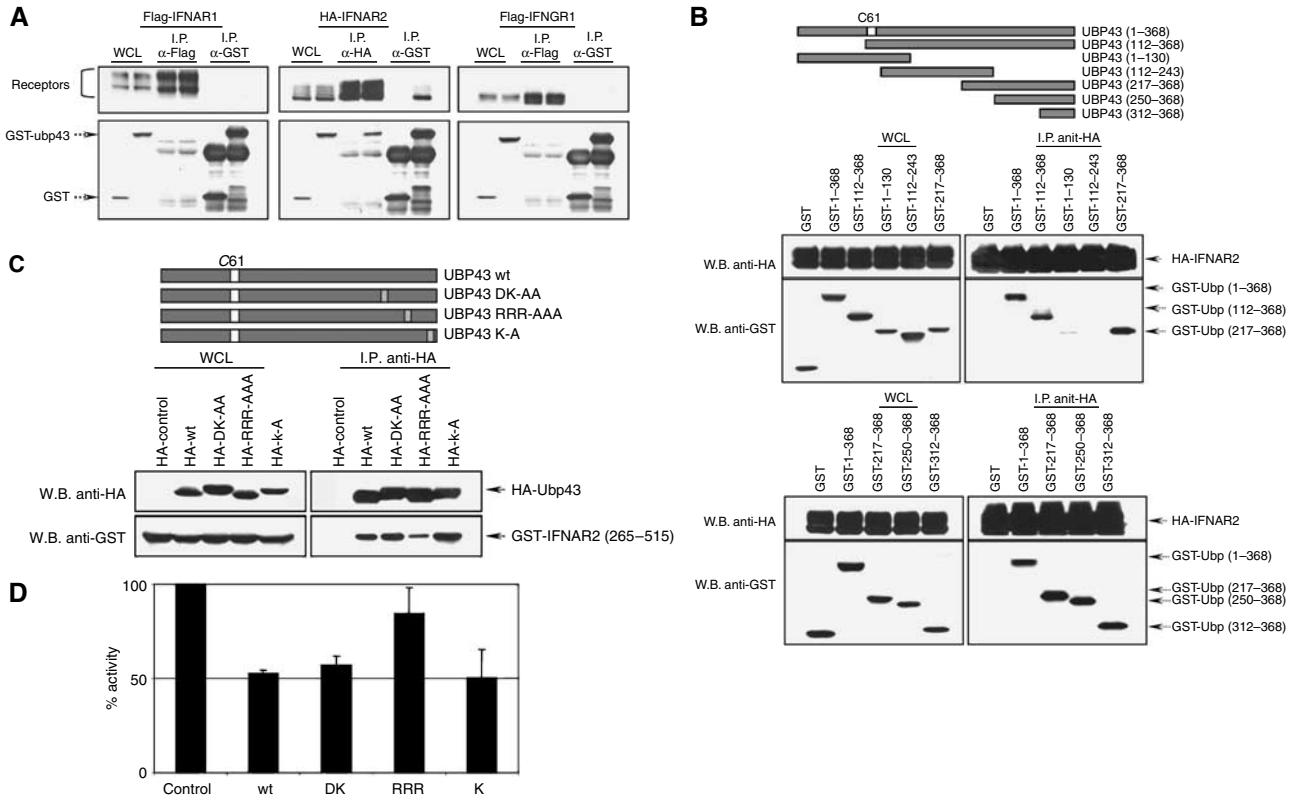

Figure 6 Ubp43 interacts with IFNAR2 receptor subunit. (**A**) 293T cells were transiently transfected with Flag-IFNAR1, HA-IFNAR2, or Flag-IFNGR1 and either GST control or GST-Ubp43. Reciprocal immunoprecipitations (I.P.) were performed using anti-Flag/HA or anti-GST antibodies. Whole-cell lysates (WCL) or immunoprecipitated complexes were subjected to immunoblotting with anti-HA antibodies (top middle panel), anti-Flag (top left & right panels) or anti-GST (bottom panel) antibodies, respectively. (**B**) Transient co-transfections of 293T cells were performed using HA-tagged IFNAR2 and various deletion mutants of Ubp43 (a.a. positions are indicated in the figure). Ubp43 deletion constructs used for this study are schematically represented in the top panel. WCL or immunoprecipitated (anti-HA) complexes were subjected to immunoblotting with anti-HA antibodies (middle panel) or anti-GST (bottom panel) antibodies, respectively. (**C**) wt Ubp43 and Ubp43 mutants: $D_{331}K_{340}$-AA, $R_{350}R_{352}R_{354}$-AAA, K_{364}-A (positions of a.a. substitutions are graphically shown on the top panel of the figure) were co-expressed with GST-IFNAR2 (a.a. 265–515) in 293T cells. I.P. were performed using anti-HA antibodies followed by immunoblotting with anti-HA antibodies (top) to verify equal loading and anti-GST-antibodies (bottom). (**D**) U3A cells were transiently co-transfected by the combination of ISRE-driven luciferase reporter plasmid, STAT1, and either vector-control, wt Ubp43, or Ubp43 mutants: $D_{331}K_{340}$-AA (DK), $R_{350}R_{352}R_{354}$-AAA (RRR) and K_{364}-A (K). At 24 h post-transfection, cells were either left untreated or treated with hIFN-α for 24 h. Luciferase activities were measured, normalized, and presented as fold increase of relative luciferase activity in IFN treated cells over the untreated controls (average of 4 independent experiments). The error bars indicate the s.d. of the mean.

To further characterize the Ubp43–IFNAR2 interaction domain and its role in IFN signaling, several charged residues potentially present on the surface of the Ubp43 312–368 fragment (based on the alignment with HAUSP deubiquitinating enzyme, for which the crystal structure is available (Hu *et al*, 2002)) were substituted to alanines. As a result, three Ubp43 mutants were made (i.e. $D_{331}K_{340}$-AA, $R_{350}R_{352}R_{354}$-AAA and K_{364}-A) within the IFNAR2 interaction region and these mutants were tested for their ability to interact with IFNAR2. Co-immunoprecipitation assays revealed that mutations at the positions 331–340 or 364 of Ubp43 did not affect its ability to interact with IFNAR2; however, binding of Ubp43 was diminished when amino-acid residues at positions 350–354 were substituted for alanines (Figure 6C, right panel, compare lanes 2 and 4), indicating that charged residues at positions 350–354 of Ubp43 were critical for its association with IFNAR2 *in vivo*. These data prompted us to examine if mutation of arginines within the 350–354 a.a. span would also reduce the potency of Ubp43 in downregulation of IFN response. To test this, U3A cells constitutively expressing wt or charged amino acids mutants of Ubp43 within the 312–368 region described above, were co-transfected with STAT1 in combination with an ISRE-driven promoter-luciferase reporter. As shown in Figure 6D, expression of either wt or $D_{331}K_{340}$-AA and K_{364}-A mutants of Ubp43 noticeably decreased the level of IFN-specific promoter activation in response to IFN in U3A cells. However, the $R_{350}R_{352}R_{354}$-AAA mutant of Ubp43 (that exhibited lower IFNAR2 binding capacity) showed substantially lower efficiency in inhibiting IFN signaling. These data provided further evidence that Ubp43 attenuates IFN signaling through the specific interaction with the IFNAR2 subunit of the receptor.

Ubp43 binds to the IFNAR2 subunit within the Box1–Box2 region and interferes with JAK1-receptor interaction

In order to determine the functional consequences of Ubp43–IFNAR2 interaction, we further identified the region of IFNAR2 that is required for Ubp43 binding. We generated constructs that expressed various fragments of the cytoplasmic portion of IFNAR2 as GST-fusions (Figure 7A, top panel). Deletion of either Box1 alone or the Box1–Box2 regions of

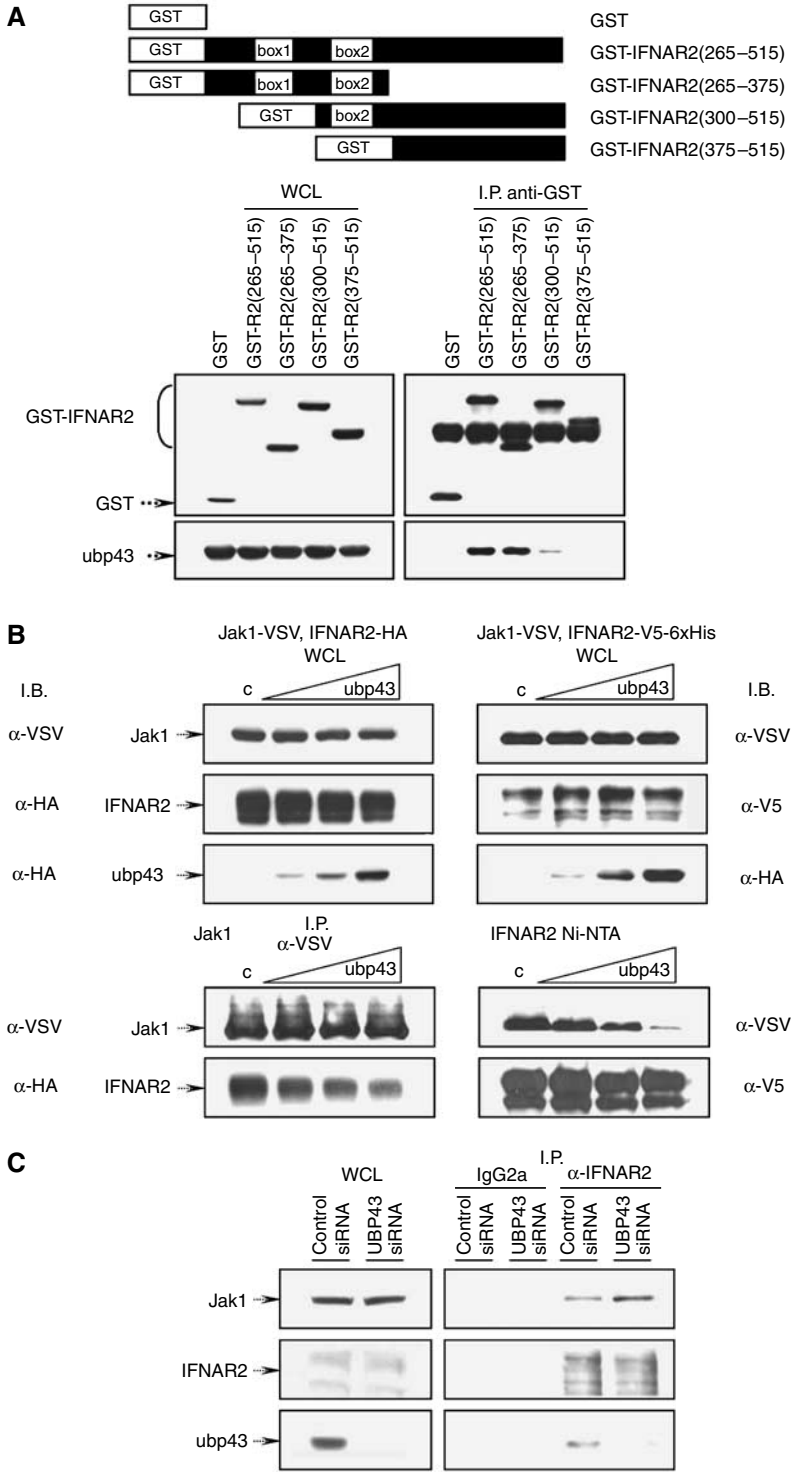

Figure 7 Ubp43 competes with JAK1 for receptor binding. (**A**) 293T cells were transiently co-transfected with HA-Ubp43 and GST or GST-IFNAR2 truncation constructs schematically presented in the top panel of the figure. Whole cell lysate (WCL) (left panels) or immunoprecipitated complexes (right panels) were subjected to immunoblotting against GST or HA-tag, respectively. (**B**) 293T cells were transiently co-transfected with JAK1-VSV and IFNAR2-HA (left) or JAK1-VSV and IFNAR2-V5-6xHis (right) in the absence or presence of increasing concentration of HA-Ubp43 followed by immunoprecipitation using antibodies against VSV-tagged JAK1 (left, bottom panels) or Ni-NTA purification of 6xHis-tagged IFNAR2 (right, bottom panels). WCL (top panels) or immunoprecipitated complexes (bottom panels) were subjected to immunoblotting with antibodies indicated in the figure. (**C**) Protein extracts from stable KT-1 transfectants expressing either control siRNA or UBP43 specific siRNA and treated with hIFN-α for 4 h were used for the immunoprecipitations with control IgG2a antibodies or with anti-IFNAR2 antibodies. WCL and immunoprecipitates were subjected to immunoblotting with anti-JAK1 (top panel), anti-IFNAR2 (middle panel), and anti-Ubp43 antibodies (bottom panel).

IFNAR2 abrogated its ability to interact with Ubp43 in co-immunoprecipitation assays (Figure 7A, bottom), indicating that the Box1–Box2 domain of the receptor is required for Ubp43 binding. Interestingly, the same domain of IFNAR2 has been reported to be a critical interaction surface for JAK1 binding (Domanski *et al*, 1997; Usacheva *et al*, 2002),

suggesting that Ubp43–IFNAR2 complex formation could compete with JAK1 receptor binding, consequently inhibiting activation and the downstream signaling cascade. To test this hypothesis, we co-expressed JAK1 and IFNAR2 in 293T cells in the absence or presence of increasing amounts of Ubp43 and performed a set of reciprocal pull down assays either for JAK1 (Figure 7B, left bottom panel) or IFNAR2 (Figure 7B, right bottom panel). As shown in the top panels (left and right) of Figure 7B, all proteins were expressed at the expected levels. We then confirmed that both JAK1 (Figure 7B, left bottom panel, lane 1) and IFNAR2 (Figure 7B, right bottom panel, lane 1) were able to interact with their respective partners in the absence of Ubp43 by co-immunoprecipitation experiments. Ubp43, however, was capable of interfering with JAK1–IFNAR2 complex formation in a dose-dependent manner (Figure 7B, left and right bottom panels, lanes 2–4). Furthermore, forced expression of Ubp43 had no effect on the interaction between JAK1 and IFNGR1 (data not shown). Given that modulation of Ubp43 level did not affect the extent of Jak–Stat signaling triggered by IFNγ (data not shown), these data are consistent with our hypothesis that Ubp43 specifically regulates type I IFN signaling via the interaction with a cognate receptor.

In order to determine whether Ubp43 confers this mode of regulation under physiological conditions, we used the RNAi approach followed by the assessment of interaction between endogenous IFNAR2 and JAK1. First, we confirmed that endogenous Ubp43 specifically interacts with endogenous IFNAR2 upon IFN stimulation in human KT-1 cells (Figure 7C, anti-IFNAR2 versus IgG2a-control immunoprecipitaion). Furthermore, knockdown of Ubp43 in KT-1 cells by Ubp43-specific siRNA increased the association between endogenous IFNAR2 and JAK1 (Figure 7C), indicating that the endogenous Ubp43 is capable of competing with JAK1 for IFN receptor binding. These data provide the genetic evidence to support the hypothesis that Ubp43-mediated titration of JAKs away from the receptor may inhibit downstream phosphorylation cascade events.

Discussion

IFNs regulate diverse cellular processes involved in cell growth, differentiation, and host defense by recruiting JAK kinases and STAT transcription factors to the specific receptors present at the cell surface (Ivashkiv and Hu, 2004; Rawlings *et al*, 2004). In order to achieve an appropriate biological response to a diverse range of extracellular stimuli, cells acquired a sophisticated and strictly controlled network involving a great variety of proteins that negatively regulate cytokine signal transduction by various means (Greenhalgh and Hilton, 2001; Yamada *et al*, 2003). Our previous studies demonstrated that *ubp43*-deficient cells are hypersensitive to type I IFN treatment, indicating that Ubp43 is a novel negative regulator of IFN signaling (Malakhova *et al*, 2003). Furthermore, Ubp43 is a specific ISG15 protease that removes the ubiquitin-like modifier ISG15 from targeted proteins and therefore controls the level of ISG15 conjugated proteins in cells (Malakhov *et al*, 2002). Indeed, *ubp43*-deficient cells have higher basal and IFN induced levels of ISGylated proteins. Naturally, the level of protein ISG15 modification was thought to be linked to the hypersensitivity of these cells to IFN. However, one of the first questions raised was whether sustained ISG15 conjugation is the cause of IFN hypersensitivity or whether the negative regulation of JAK–STAT signaling by Ubp43 is mediated by an ISG15-independent mechanism. Analyses of stable *ubp43−/−* MEF cell lines reconstituted with either ISG15 activating enzyme Ube1L siRNA or Ubp43 demonstrated that the inhibition of ISG15 conjugation by knocking down Ube1L in *ubp43−/−* cells cannot reverse the IFN hypersensitive phenotype of *ubp43*-deficient cells. On the other hand, the reintroduction of wt or isopeptidase active site mutated Ubp43 protein into *ubp43−/−* MEFs can effectively inhibit IFN sensitivity to a level comparable with wt cells. Such an observation is also confirmed in two different human cell lines used in this study. When either wt or the enzyme active site mutated Ubp43 is ectopically expressed in U3A or KT-1 cells, a specific inhibition of STAT1 phosphorylation and subsequent activation of ISGs in response to IFN is observed. These results clearly demonstrate that Ubp43 downregulates the cellular response to IFN independently of its enzymatic activity towards ISG15. These data were also confirmed in *ubp43−/− ube1L−/−* double knockout mice, where MEF cells deficient for both Ubp43 and ISG15 conjugation retained the same level of IFN sensitivity as was observed in *ubp43*-knockout cells (Kim *et al*, 2006).

In recent years, several proteins within the JAK–STAT pathway were found to be targets for ISG15 modification, including JAKs and all STAT family members (Malakhov *et al*, 2003 and our unpublished data), implying that modification of these proteins by ISG15 may influence their structure, function, and overall signaling in general. Our current data suggest that high accumulation of ISG15 conjugates in *ubp43*-deficient cells is most likely a result but not a main cause of the IFN hypersensitivity observed in these cells.

Since the duration of IFN signaling is known to be controlled at multiple levels, including receptor proteolysis and activity of kinases and phosphatases, we further examined which particular step of the IFN signaling cascade involves Ubp43. We found that Ubp43 inhibits JAK–STAT signaling at the level of the IFN receptor, but is not involved in the control of receptor proteolysis or in sustaining the level of the IFN receptor at the cellular surface. Importantly, Ubp43 is capable of inhibiting the activation of the receptor-associated kinases—JAK1 and TYK2. While Ubp43 deficiency results in sustained phosphorylation of these JAKs in response to IFN, the forced expression of either wt or active site mutated Ubp43 reduces the phosphorylation level of these kinases and their downstream phosphorylation and transcriptional events as well as resistance to viral infection.

The ability of Ubp43 to attenuate the activity of JAKs suggests that it may associate with one or more components of the type I IFN-receptor complex and both JAKs and the receptor subunits represent potential targets for Ubp43 action. It is tempting to hypothesize that the direct binding of Ubp43 to JAKs may cause inhibition of its tyrosine kinase activity. However, further investigation did not support this theory since Ubp43 was not able to inhibit JAK1 or TYK2 activities that were auto-induced by constitutive overexpression of the respective kinases (data not shown). In addition, Ubp43 was found to act specifically for type I IFN signaling and did not inhibit the IFN-γ, IL-6, or IL-12 signaling pathways; even though all tested cytokines required JAK1 or

TYK2 for signal transduction. These results suggested that inhibition of JAK phosphorylation by Ubp43 depended on the molecular specifics of type I IFN signaling and was likely to be achieved through an interaction between Ubp43 and type I IFN specific components of the IFN-receptor complex. Indeed, further investigation revealed that Ubp43 specifically interacted with the IFNAR2 chain of the type I IFN receptor, but not with IFNAR1 or IFNGR1. The physiologic effects of disrupting the Ubp43–IFNAR2 interaction were also investigated. The most important result of these studies is the identification of an Ubp43 mutant that cannot efficiently bind IFNAR2 and impair IFN-dependent induction of an ISRE-linked reporter gene. This result strongly suggests that the Ubp43–IFNAR2 interaction is critical for Ubp43-dependent inhibition of JAK–STAT signaling. Additional studies will be required to delineate the structure-function relationships of this interaction.

In order to determine the functional consequences of the Ubp43–IFNAR2 interaction, we identified the region of IFNAR2 required for Ubp43 binding. We found that the Ubp43 binding site is located in the membrane-proximal region of IFNAR2, covering the Box1–Box2 motifs of the receptor. The same domain of IFNAR2 is essential for the interaction with JAK1 (Domanski *et al*, 1997; Usacheva *et al*, 2002), suggesting that Ubp43 could compete with JAK1 for receptor binding, consequently inhibiting its activation and the downstream intracellular signaling. Indeed, our experimental data from co-expression experiments demonstrate that Ubp43 inhibits the formation of the JAK1–IFNAR2 complex in a dose-dependent manner, suggesting that titration of JAKs away from the receptor may account for the observed inhibition of the downstream phosphorylation cascade and therefore represent the putative mode of Ubp43 action in the negative regulation of IFN signaling. This hypothesis has been corroborated by genetic evidence showing that knockdown of endogenous Ubp43 promotes the interaction between endogenous JAK1 and endogenous receptor subunit. It is also possible that, as in the case of SOCS proteins (Greenhalgh and Hilton, 2000; Yasukawa *et al*, 2000; Kumar *et al*, 2003; Shuai and Liu, 2005), there are multiple roles for Ubp43 in regulating signal transduction, including its function in regulating protein ISGylation. Indeed, recent report clearly point to the role of ISG15 in the antiviral defense (Lenschow *et al*, 2005). Further studies, such as knockin of the enzymatically inactive *ubp43* into the *ubp43* locus, will provide additional information about different aspects of Ubp43 in cellular innate immune responses.

The ability of Ubp43 to inhibit signal transduction and ultimately the biological response to IFN suggests that, like other known negative regulators of signal transduction, such as phosphatases (SHP-1, SHP-2, TC45, and PTP1B), SOCS and PIAS proteins, Ubp43 plays an important role in controlling the magnitude and/or duration of cellular responses to type I IFN. The evidence provided here, as well as our previous findings, suggests that Ubp43 acts in a classic negative feedback loop for IFN signal transduction. Like other genes such as SOCS, Ubp43 is induced by IFNs through the activation of the JAK–STAT pathway (Li *et al*, 2000; Kang *et al*, 2001). Once induced and expressed, Ubp43 can directly bind to the IFN receptor and inhibit the receptor interaction with JAK, thereby reducing the phosphorylation of the receptor and STATs and suppressing signal transduction and downstream biological responses. Ubp43 deficient mice are highly resistant to viral/bacterial infection and cancer development in several experimental mouse models (Ritchie *et al*, 2004; Kim *et al*, 2005;Yan and Zhang, data in preparation). It is of added importance that, unlike phosphatases, SOCS and PIAS that affect cellular responses to many cytokines, Ubp43-mediated inhibition is specific for the type I IFN signaling. Given that specificity and the fact that type I IFN are widely used as antiviral, anticancer, and immunomodulatory agents in therapy of chronic viral infections, malignancies, and relapsing-remitting multiple sclerosis, Ubp43 appears to be a promising therapeutic target for improving the benefits of treatment.

Materials and methods

Details provided as Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank Drs Ernest Borden, James Darnell, Ueli Gubler, John Krolewski, Sandra Pellegrini, Lawrence Pfeffer, George Stark, and Gilles Uze for antibodies, cell lines, and DNA constructs, and members of Zhang lab for valuable discussions. This work was supported by research funding from National Institutes of Health (CA079849 to DEZ and CA092900 to SYF) and Department of Defense (DAMD17-03-1-0269 to DEZ). The Stein Endowment Fund has partially supported the departmental molecular biology service laboratory for DNA sequencing and oligonucleotide synthesis. This is manuscript 17422-MEM from The Scripps Research Institute.

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Supplementary information

Materials and Methods

Plasmid construction. Constructs for pCAGGS-6xHis-mISG15, pCAGGS-HA-UBE1L, pFlagCMV2-UbcM8 (Kim et al., 2004) and pcDNA3-IFNAR1-Flag (Kumar et al., 2003) were previously described. Human VSV-tagged JAK1 and TYK2 (pRcCMV) expression constructs were provided by Sandra Pellegrini (Paris, France). Flag-STAT1 expression constructs were provided by Jim Darnell (New York, NY, USA). Control siRNA (primer set: 5'-gggagatctcaaggtcgggcaggaagagggcctatttcc-3'; 5'-ggggaattcaaaaagacctcggtgataccaaggtctcttgaaccttggtatcaccgaggtcggtgtttcgtcctttc cacaagatatataa-3';) (in MSCV-PGKpuro), hUbp43 specific siRNA (primer set: 5'-gatccccaggagaagcattgttttcaaattcaagagatttgaaaacaatgcttctccttttta-3'; 5'-agcttaaaaa aggagaagcattgttttcaaatctcttgaatttgaaaacaatgcttctcctggg-3') (in pRetro-puro) and Ube1L-specific siRNA (primer set: 5'-tttggatcccaaggtcgggcaggaagagggcctatttcc-3'; 5'-tttgaatt caaaaaggatgatgacagcaacttctctcttgaagaagttgctgtcatcatccggtgtttcgtcctttccacaagatat ataa-3') (in MSCV-PGKpuro) constructs were generated by PCR and subcloned into respective vectors under control of the U6 promoter. Correct constructs were confirmed by sequencing. VSV-tagged mIFNAR1 was received from Gilles Uze (Montpellier, France) and subcloned into pcDNA3.1 vector (Invitrogen). hIFNGR1 cDNA was PCR amplified from M426 human lung fibroblast cDNA library (using the following primers: (F) 5'- ataggatccaccatggctctcctctttctcctacc-3' and (R) 5'-gtagtctcgagctgaaaattctttggaatcttctgtttgg-3'), digested with BamHI /XhoI and ligated in frame into pCDNA3-C-3X-FLAG vector that contained three Flag repeats before the stop codon. HA-hIFNAR2 expression construct was received from John Krolewski (Irvine, CA, USA). The GST-tagged IFNAR2 constructs containing the cytosolic portion of the receptor (a.a. 265-515) and IFNAR2 truncations: a.a. 265-375, 300-515 and 375-515 were generated by inserting a PCR fragment encompassing a full length hIFNAR2 cDNA into the pEBG vector in frame with GST. V5-6xHis-tagged hIFNAR2 was generated by inserting a full length hIFNAR2 cDNA into the pcDNA6-V5-6xHis vector (Invitrogen) in frame with V5 and 6xHis. V5-tagged mUbp43wt or mUbp43^{C61S} were in the pcDNA6 vector (Invitrogen) and were previously described (Malakhov et al., 2002). The N-terminally HA-tagged Ubp43 constructs including wt Ubp43, c61s Ubp43, $D_{331}K_{340}$-AA, $R_{350}R_{352}R_{354}$-AAA and K_{364}-A mutants were generated by PCR using a full length mUbp43 cDNA as a template and inserting it into the pcDNA3-HA vector (Invitrogen). Retroviral constructs containing untagged mUbp43wt or mUbp43^{C61S} were generated in pLPCX vector (Clontech). The GST-tagged Ubp43 constructs including full length Ubp43 (1-368), deletion fragments encompassing amino acids 1-130, 112-368, 112-243, 217-368,250-368, and 312-368 were generated by PCR and subcloned into the pEBG vector in frame with GST. All mutant constructs were confirmed by sequencing.

Cell culture and transfections. HEK293T cells, MEFs, and U3A cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with 10% fetal bovine serum (FBS, HyClone) and 2 mM L-glutamine (Invitrogen). Human KT-1 cells were cultured in RPMI medium (Invitrogen) with 10% FBS and 2 mM L-glutamine. Cycloheximide was purchased from Sigma and used at a concentration of 20 μg/ml. Human IFN-α2 (Roferon-A) was from Roche Pharmaceuticals and was used at a concentration of 1,000 U/ml for all cell treatments. Mouse IFN-β was purchased from ICN Pharmaceuticals and used at a final concentration of 1,000 U/ml.

For small-scale transfections, cells were grown on six-well plates and transfected using PolyFect reagent (QIAGEN) according to the instructions. For large-scale transfections, cells were plated on 10-cm dishes and transfected by using calcium phosphate precipitation as described previously (Zhang et al., 1994). Immortalized *ubp43+/+* and *ubp43-/-* MEFs were described previously (Malakhov et al., 2003). Stable pools of MEF, U3A, and KT-1 cells were made after retroviral infection with the respective constructs by selection with 2 μg/ml of puromycin (Calbiochem).

Antibodies and Reagents. Antibodies against Flag, V5, hIFNAR1, tubulin, STAT2 (Sigma), HA (Covance), VSV (Bethyl Lab), JAK1 (pYpY1022/1023), JAK1, TYK2 (pYpY 1054/1055), STAT1 (pY701) (Cell Signaling), STAT 1, STAT3, hIFNAR2 (Santa Cruz), TYK2 (BD Biosciences), and hIFNAR2 (PBL) were purchased from the respective manufacturers. Rabbit anti-mouse Ubp43 and anti-mouse ISG15 polyclonal antibodies were described previously (Malakhov *et al.*, 2002). Rabbit anti-hUbp43 polyclonal antibodies were generated against specific peptide (aa 37-54) of human Ubp43 protein and affinity purified by using immobilized antigen. Mouse monoclonal antibodies against human ISG15 were a generous gift from Ernest Borden (Cleveland, OH, USA). Sodium orthovanadate (Fisher) was used at 2 mM. Proteasome inhibitor MG132 (Sigma) was used at 10 μM.

Immunoprecipitation and Western blot analyses. For immunoprecipitation, cells were lysed in 1x TBS buffer [50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40] by sonication. Immunocomplexes from 0.5-1 mg of total cell lysates were precipitated with a mixture of protein A-agarose and protein G-agarose (Amersham Biosciences). Immunoprecipitates were washed three times with the same buffer, boiled in SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer, and subjected to SDS-PAGE followed by Western blotting. Western blotting was performed as described previously(Malakhova et al., 2003). Ni-NTA purification of 6xHis-tagged proteins was performed under native conditions according to the instructions, suggested by the manufacturer (QIAGEN).

FACS analysis. Cells (1×10^7/ml) were incubated with primary antibodies (with manufacturer suggested dilutions) on ice for 1 h, following by staining with FITC-labeled secondary antibodies and analyzed using FACSCalibur (BD Biosciences).

Viral infection. VSV anti-viral assay was performed as reported previously (Wong et al., 2001).

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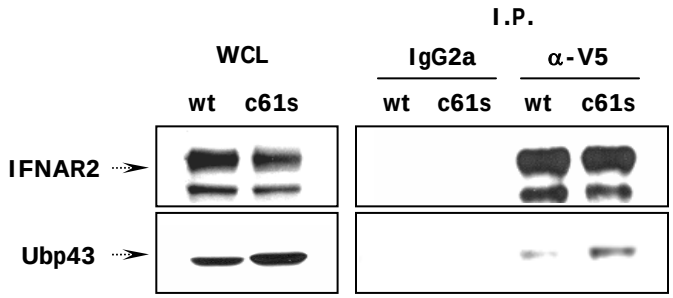

Figure S1. Both wt and active site mutated Ubp43 interact with IFNAR2. 293T cells were transiently transfected with IFNAR2-V5-6xHis and either HA-Ubp43 or HA-Ubp43C61S. Immunoprecipitations were performed using control IgG2a or anti-V5 antibodies. Whole cell lysates (WCL) or immunoprecipitated complexes were subjected to immunoblotting with anti-V5 antibodies (*top panels*), or anti-HA antibodies (*bottom panel*), respectively.

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Article title: Ubp43 regulates BCR-ABL leukemogenesis via the Type I interferon receptor signaling

Short title: Ubp43 regulates BCR-ABL leukemogenesis

Ming Yan[1], Jiann-Kae Luo[1], Kenneth J. Ritchie[1,#], Ruibao Ren[2], Dong-Er Zhang[1,*]

[1]Division of Oncovirology, Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA 92037, [2]Rosenstiel Basic Medical Sciences Research Center, Department of Biology, Brandeis University, Waltham, MA 02454

[#] Current address: Cancer Research UK Molecular Pharmacology Unit, Ninewells Hospital and Medical School, Dundee, DD1 9SY, United Kingdom

Author contribution: MY performed majority of experiments, analyzed data and wrote the paper. JKL performed significant amount of experiments and wrote the paper, KJR helped with generating UBP43 knockout mice and edited the paper, RR provided Mig-p210 construct and bone marrow transplantation method, DEZ designed the experiments and wrote the paper.

This work was supported by research funding from Department of Defense (DAMD17-03-1-0269) and National Institutes of Health (CA102625). The Stein Endowment Fund has partially supported the departmental molecular biology service laboratory for DNA sequencing and oligonucleotide synthesis.

Total character counts: 200 (Abstract), 3886 (Text)

[*] Corresponding Author:
Dong-Er Zhang, Ph.D.
MEM-L51
The Scripps Research Institute
10550 North Torrey Pines Road
La Jolla, CA 92037
Tel: 858-784-9558
Fax: 858-784-9593
Email: dzhang@scripps.edu

Abstract

Interferon (IFN) signaling induces the expression of interferon responsive genes and leads to the activation of pathways that are involved in the innate immune response. Ubp43 is an ISG15 specific isopeptidase, the expression of which is activated by IFN. Ubp43 knockout mice are hypersensitive to IFN-α/β and have enhanced resistance to lethal viral and bacterial infections. Here we show that in addition to protection against foreign pathogens, Ubp43 deficiency increases the resistance to oncogenic transformation by BCR-ABL, the causative agent of chronic myeloid leukemia (CML). BCR-ABL viral transduction/transplantation of wild-type bone marrow cells results in the rapid development of a CML-like myeloproliferative disease, in contrast a significantly increased latency of disease development is observed following BCR-ABL viral transduction/transplantation of Ubp43 deficient bone marrow cells. This resistance to leukemic development is dependent on Type I IFN (IFN-α/β) signaling in Ubp43 deficient cells. Increased levels of Type I IFN are also detected in the serum of CML mice. These results suggest that inhibition of Ubp43 can potentiate the response to increased endogenous IFN levels in innate immune responses against cancer development, indicating that pharmacological inhibition of Ubp43 may be of benefit in cancers and others diseases in which interferon is currently prescribed.

Introduction

Innate immune responses play critical roles in the inhibition of cancer development.[1] One of the key components of the innate immune response is activation of the type I interferon (IFNα/β) signaling pathway.[2;3] Binding of type I IFNs to their receptor (IFNAR) triggers the phosphorylation of receptor-associated Jak1 and Tyk2 kinases.[4;5] These kinases then phosphorylate STAT1 and STAT2, leading to the activation of downstream signal transduction pathways.[6-9] Furthermore, a family of suppressors of cytokine signaling (SOCS) and several protein tyrosine phosphatases negatively regulate the STAT signaling pathway.[10-12] Defects in such regulators may result either in the loss of response or a hyper-response to IFN stimulation.

Type I IFN signaling triggers the expression of hundreds of IFN stimulated genes (ISGs).[13;14] Among these is the ISG15 deconjugating enzyme Ubp43 (Usp18).[15-19] ISG15 is a ubiquitin like modifier whose expression and conjugation to other proteins (ISGylation) is strongly increased upon Type I IFN stimulation.[20;21] Ubp43 deficient cells accumulate higher levels of ISGylated proteins and are hypersensitive to Type I IFN treatment, as evidenced by the enhanced and prolonged activation of STAT phosphorylation in these cells.[22;23] Furthermore, Ubp43 knockout mice show a higher resistance to viral and bacterial infection,[24;25] indicating an important role for Ubp43 in the regulation of IFN signal transduction. Recently, using cells with different levels of protein ISGylation and Ubp43 expression, we demonstrated that UBP43 is a novel negative regulator of Type I interferon signaling and this function is independent of Ubp43 isopeptidase activity against ISG15 conjugates.[26;27]

Type I IFNs suppress cell proliferation and promote apoptosis,[28] as such they have been used in the clinical treatment of several cancers, including leukemia.[29] A specific example is in the treatment of chronic myeloid leukemia (CML), where IFN was the primary choice before imatinib mesylate became available.[30-32] In nearly all cases of CML, patients carry a reciprocal translocation between chromosomes 9 and 22.[33;34] This results in a fusion protein consisting of the N-terminal portion of BCR joined to most of the ABL tyrosine kinase. The chimeric BCR-ABL tyrosine kinase is constitutively activated as a result of the oligomerization domain provided by BCR. The tyrosine kinase activity of BCR-ABL activates several signaling intermediates, such as Ras, Akt, Stat5, and p38, which in turn triggers deregulated growth and proliferation of the myeloid progenitors and promotes CML development.[6;35-37]

Expression of BCR-ABL in mouse bone marrow cells is sufficient for the rapid onset of a CML-like myeloproliferative disease, characterized by splenomegaly and high white blood cell counts.[38-40] Given the hypersensitivity of Ubp43$^{-/-}$ mice to IFN and the efficacy of IFN in CML treatment, we investigated whether genetic ablation of UBP43 function resulted in delayed oncogenic transformation in vivo. Here, we report that, compared to wild-type controls, transplantation of BCR-ABL expressing Ubp43$^{-/-}$ bone marrow cells into mice results in a greatly prolonged latency period in the development of the CML-like myeloproliferative disease. This resistance to leukemia development is dependent upon activation of the Type I IFN signaling pathway, suggesting that inhibition of Ubp43 promotes an enhanced response to endogenous IFN levels in innate immune responses against cancer development.

Materials and Methods

Animals and cells. The generation of Ubp43$^{-/-}$ mice and immortalized MEFs have been described previously.[22;23;26] Generation and culture of KT-1 cells with stable expression of UBP43 shRNA have been described previously.[26] IFN-α/β receptor R1 subunit knockout mice (Ifnar1$^{-/-}$) in the C57 background were kindly provided by Jonathan Sprent (The Scripps Research Institute)[41] and bred with the Ubp43$^{-/-}$ mice to generate Ubp43$^{-/-}$/Ifnar1$^{-/-}$ double knockout mice. Wild type C57BL/6J congenic strain C57/B6.SJL Pep3b-BoyJ mice were purchased from Jackson Laboratory. Mice were housed in a pathogen-free facility and procedures were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

BCR-ABL retrovirus and retroviral transduction. 293T cells were co-transfected with MCV-ecopac and either MigR1 or Mig-p210 at a 1:1 ratio by the calcium phosphate precipitation method. Media was changed 24 hours after transfection and the retroviral supernatants were harvested the following day and filtered through a 0.45 μm filter. In most experiments the retrovirus was used immediately to insure a high viral titer. To infect bone marrow cells, 2 ml retroviral supernatant supplemented with 8 μg/ml of polybrene (Sigma) was added to 1 x 10^6 cells in a six-well plate and centrifuged at 1,400x g for 120 min at 32 C. The supernatant was then removed and the cells were resuspended in the bone marrow cell culture medium. Cells were incubated overnight at 37^{o}C in 5% CO_2 before performing a second round of infection. The efficiency of

retroviral transduction was determined on the basis of green fluorescence by flow cytometry 24 hours after the second round of infections.

Bone marrow transplantation. All recipient mice (6-8 weeks old) were lethally irradiated with 900 rads in a split dose separated by at least 3 hours. Ubp43$^{-/-}$, their Ubp43$^{+/-}$ and wild-type littermates, as well as Ubp43$^{-/-}$/Ifnar1$^{-/-}$ donor mice were injected with 100 mg/kg body weight of 5-fluorouracile (5-FU) (Sigma). Five days after 5-FU injection, bone marrow cells were harvested from these mice and spin-infected with Mig-p210 or MigR1 retrovirus as described. 24 hours after the second round of infections, the bone marrow cells (4 x 10^5 cells) were transplanted into the recipient mice by tail vein injection. Mice were maintained in sterilized cages for 3 weeks on acidified water (pH 4.0). Upon leukemia development, moribund mice were euthanized.

Murine IFN injection. Where applicable, bone marrow transplantation recipients were injected subcutaneously with 18,000 units mIFNβ (MP Biomedicals) per day starting at day 17 (for UBP43$^{+/+}$ donor cells) or day 28 (for UBP43$^{-/-}$ donor cells) post transplantation.

Hematological analysis. Two μl of blood was diluted in 78 μl of Türk's solution (0.01% crystal violet and 3% glacial acetic acid) and white blood cell counts were performed under microscopic observation. ACCUSTAIN™ Wright stain and ACCUSTAIN™ Giemsa stain solutions (Sigma) were used to stain peripheral blood smears as well as spleen and bone marrow cytospin slides following the two step staining protocol from the

manufacturer. Differential counts of blood and bone marrow cells were obtained by counting 200 nucleated cells for each sample.

Histology. Spleen and liver samples were fixed with 4% paraformaldehyde in PBS overnight at room temperature and stored at 4^{o}C. Paraffin-embedded sections were cut to a 5 μm thickness and stained with Hematoxylin and Eosin.

Apoptosis Assay. The percentage of apoptotic cells was analyzed by FACS after staining cells with the Annexin V-PE apoptosis detection kit (BD Pharmingen) according to manufacturer's protocol. Flow cytometry was performed with FACSCalibur (BD immunocytometry).

Murine IFN bioassay. Serum levels of IFN were measured using an *in vitro* biological assay for protection against the cytopathic effect of VSV on murine L929 cells. Briefly, two fold serial dilutions were performed in 96 well plates using RPMI medium containing 2 mM glutamine, 100 units/ml penicillin/streptomycin, and 10% FBS. 3.5 x 10^5 L929 cells were added to each well and cultured for 24 hours at 37^{o}C in a 5% CO_2 atmosphere. VSV was then added to each well to a final MOI of 0.1. Protection against VSV-mediated cytopathic effects was then evaluated at 48 hours after addition of the virus by MTT assay.[42;43] Murine IFN-β (ICN Biomedicals) was used as a standard.

Isolation of RNA and Northern blot analysis. Spleen cells were harvested from the BCR-ABL induced leukemic and MigR1 control infected mice. RNA was isolated by the

RNABee extraction kit (Tel-Test Inc, Friendswood, TX). Ten μg of total RNA for each sample was electrophoresed on a 1% agarose gel containing 0.22 mol/L formaldehyde. The RNA was transferred to Hybond-N nylon membranes (Amersham) and hybridized to ^{32}P-dATP radiolabeled probes for murine Ubp43 cDNA.

Immunoblotting. Cells were harvested in a buffer containing 50 mM Tris, pH 7.6, 3 M NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate and supplemented with protease inhibitor cocktail (Sigma) and 1 mM each of NaF and $NAVO_3$ to inhibit protein phosphatase activity. Lysates were cleared by centrifugation at 18,000x g for 10 min and western blotting performed as described previously.[44] BCR-ABL was detected with the anti-ABL antibody AB-3 (Oncogene Research Products). Anti-TRAIL and anti-cytochrome C antibodies are from BD Biosciences and anti-caspase 3 antibodies are from Cell Signaling Technologies.

Statistical analyses. Statistical significance of the survival times of bone marrow transplantation recipients was calculated by chi squared test using the program Prism 4 (Graphpad Software Inc.). Statistical significance of the apoptosis studies in KT-1 cells was analyzed using the two-tailed Student's t-Test.

Results

Ubp43 deficient cells can be used in retroviral transduction and bone marrow transplantation assays. Targeting BCR-ABL expression to the murine hematopoietic cell via the MSCV-based vector has been shown to cause mice to succumb rapidly to a CML-like myeloproliferative disease.[39;40] We decided to investigate whether Ubp43 expression affects the development of CML using retrovirus mediated BCR/ABL expression and bone marrow transplantation (BMT) studies. To investigate if Ubp43 deficient cells could be virally transduced and transplanted, bone marrow cells were collected from 5-FU treated Ubp43 deficient mice and their control littermates. Since $Ubp43^{+/+}$ and $Ubp43^{+/-}$ cells responded similarly to IFN, both cell types were used as controls based upon their availability. Two days after infection with MSCV-IRES-EGFP (MigR1) vector control virus, bone-marrow cells were transplanted into γ-irradiated wild type recipient mice. Similar percentages of retrovirally infected $Ubp43^{+}$ and $Ubp43^{-}$ cells ($EGFP^{+}$) were detected in recipient mice over a two month period after BMT (Supplemental Fig. 1). These results indicate that both $Ubp43^{+}$ and $Ubp43^{-}$ hematopoietic cells can be infected with MSCV-based retrovirus and repopulate recipient mice at equivalent efficiencies.

Murine recipients of Ubp43 deficient cells are resistant to BCR-ABL induced CML like disease. To examine the role of Ubp43 in BCR-ABL induced leukemia development, bone marrow cells from $Ubp43^{+/+}$, $Ubp43^{+/-}$, and $UBP43^{-/-}$ mice were transduced either with MigR1 vector retrovirus or BCR-ABL expressing retrovirus

MSCV-BCR-ABLp210-IRES-EGFP (Mig-p210) and transplanted into wild type recipient mice. Significantly all mice transplanted with Mig-p210 infected Ubp43$^{+/+}$ and Ubp43$^{+/-}$ cells developed a CML-like disease and showed a high percentage of EGFP^{+} cells in their peripheral blood within 28 days (Fig. 1A). These mice also showed a significant increase of CD11b^{+}/Gr-1^{+} myeloid cells in their bone marrow and peripheral blood (data not shown), splenomegaly, Ubp43 expression in the spleen (Fig. 1B), and a fatal myeloproliferative disease by 33 days post BMT in agreement with other similar studies (Fig. 1C).[40;45;46] Transduction of the empty MigR1 vector control virus did not cause disease. In contrast recipients of BCR-ABL transduced Ubp43$^{-/-}$ bone marrow cells showed a substantially increased survival rate, with all of mice alive at 65 days post BMT and over 60% alive 130 days post BMT (Fig. 1C). As shown in figure 1D, the WBC count in the peripheral blood also showed significant differences. In mice transplanted with BCR-ABL expressing Ubp43$^{+/+}$ cells, a rapid increase in WBC number was observed. In contrast, recipients of Mig-p210 transduced Ubp43$^{-/-}$ bone marrow cells showed comparable WBC counts to recipients of MigR1 transduced bone marrow cells. It is interesting to note that some of the mice with Mig-p210 transduced Ubp43$^{-/-}$ cells either temporarily or consistently showed a relatively high percentage (greater than 40%) of EGFP^{+} (BCR/ABL expressing) cells in their peripheral blood, but did not develop any further symptoms of a CML like disease (Fig. 1A). This suggests that the lack of Ubp43 expression suppresses the expansion of BCR-ABL expressing cells in the peripheral blood.

Pathologic changes in mice succumbing to BCR-ABL induced leukemia. All mice transplanted with BCR-ABL expressing Ubp43^{+} bone marrow cells developed a CML-like myeloproliferative disease. In contrast less than 40% of mice with BCR-ABL expressing Ubp43^{-} cells developed a similar disease after a much longer latency period. Moreover, the severity of the CML-like disease in these two groups of mice also showed several differences. Mice receiving Mig-p210 transduced Ubp43^{+} bone marrow cells displayed the characteristic of high white blood cell counts and splenomegaly typical of this CML model (Fig. 2A). In contrast recipients of Mig-p210 transduced Ubp43^{-} bone marrow showed much lower WBC counts and spleen weight. The leukemic cell infiltrations in the liver and spleen were also histologically examined (Fig. 2B). Diseased mice with BCR-ABL expressing Ubp43^{-} cells were found to have significantly reduced leukemic cell infiltration of liver and spleen.

The expression and phosphorylation of BCR-ABL are not affected by the presence of Ubp43. The BMT experiments indicated that loss of Ubp43 confers a significant survival advantage by counteracting the oncogenic properties of BCR-ABL. Since Ubp43$^{-/-}$ cells are hypersensitive to IFN and have increased levels of ISG15 modified proteins, we speculated that the molecular mechanism of reduced oncogenic development may be direct down regulation of BCR-ABL expression through IFN signaling. The low infiltration of BCR-ABL positive cells into the spleen and liver of leukemic mice with Mig-p210-Ubp43$^{-/-}$ cells however prevented elucidation of BCR-ABL levels in these mouse tissues. Instead we performed similar retroviral transductions with MigR1 and Mig-p210 into immortalized MEFs from wild-type and Ubp43$^{-/-}$ mice. We also

reconstituted Ubp43 expression into immortalized Ubp43$^{-/-}$ MEFs as an additional control (Ubp43reconst). However, there was no gross change in BCR-ABL levels and phosphorylation after 24 hours treatment with 500 U/ml IFN-β for any of the cell types, indicating that neither IFN signaling nor the absence of Ubp43 inhibited the expression and the phosphorylation of the BCR-ABL protein (Supplemental Fig. 2). Thus, Ubp43 inhibition of BCR-ABL driven oncogenic development occurs downstream of the BCR-ABL fusion protein.

CML mice have increased Type I IFN in their sera. Ubp43 deficient cells are hypersensitive to IFN-α/β treatment and display a strong apoptotic response upon IFN treatment.[23] It has been reported previously that human CML patients have increased production of IFN-α.[47] Therefore, we hypothesized that increased Type I IFN production during leukemogenesis and the enhanced apoptosis of Ubp43 deficient cells upon IFN stimulation could explain the resistance to CML development observed in BCR-ABL expressing Ubp43 deficient cells. To test this hypothesis, we first analyzed the serum level of Type I IFN. The basal level of IFN in the serum of normal healthy mice is 10 – 20 units/ml. Similar levels of IFN were detected in mice transplanted with retroviral vector transduced bone marrow cells (Fig. 3). However a four-fold increase of IFN was detected in mice that have developed the CML-like myeloproliferative disease (Fig. 3).

Increased apoptotic rate in Ubp43-deficient BCR/ABL^{+} cells in vivo. In consideration of IFN production being increased in CML mice, we examined the percentage of apoptotic cells in mice transplanted with BCR-ABL expressing Ubp43^{+} or

Ubp43$^-$ donor cells. For this analysis we utilized the fact that all BCR-ABL$^+$ cells also expressed EGFP. Three to four weeks after BMT, and before emergence of disease symptoms, less than 2% of early apoptotic (Annexin V$^+$/7-AAD$^-$) and late apoptotic/necrotic (Annexin V$^+$/7-AAD$^+$) cells were detected in the blood of mice transplanted with BCR-ABL expressing wild type donor cells (Fig. 4). In contrast, over 20% of the EGFP+ blood cell population was apoptotic in mice transplanted with BCR-ABL expressing Ubp43$^-$ donor cells (Fig. 4). Increased apoptosis, although to a lesser degree, was also detected in the EGFP$^-$ cells in these mice. These results support the hypothesis that the enhanced IFN sensitivity observed in Ubp43 deficient cells leads to the prevention of CML development.

Knockdown of UBP43 sensitizes cells to the apoptotic effects of Type I IFN. The increased apoptosis in the UBP43$^-$ cells, together with our observation of IFN upregulation upon CML development, suggested a connection between these phenotypes and prompted us to examine whether this effect can be reproduced in the CML patient derived KT-1 cell line.[48] Pools of KT-1 cells stably expressing UBP43 shRNA show a significant increase in apoptosis upon IFN treatment compared to cells expressing a control shRNA (Fig. 5A). Furthermore, UBP43shRNA expressing cells activated the apoptotic response even with a 10-fold reduction in the IFN concentration, at which there is no significant increase in apoptosis for the control shRNA expressing cells (Fig. 5B). The increase in apoptosis upon IFN treatment can be attributed to the increased expression of apoptotic genes in UBP43 deficient cells. Microarray analysis (Zou et al., data in preparation for publication) had shown several apoptosis related

genes to be upregulated in UBP43$^{-/-}$ macrophages upon IFN treatment, including cytochrome-c, caspase-3, and TRAIL. No clear increase in cytochrome-c protein expression was observed in the KT-1 cell lines upon IFN treatment (Fig. 5C). However, in the UBP43 shRNA expressing lines, there was a significant increase in the expression of TRAIL and caspase 3 proteins, as well as cleavage of caspase 3 into its active form between 24 and 48 hours after IFN treatment, concomitant with the observed increase in apoptosis.

Response of Ubp43-deficient BCR/ABL^{+} recipient mice to IFN treatment. If the UBP43^{-} donor cells are more sensitive to IFN induced apoptosis as a result of the increased serum IFN levels associated with CML development, this would explain why these recipient mice show prolonged survival compared to wild-type recipients. To further test the role of IFN in disease progression in these mice, we examined the effects of daily subcutaneous injections of murine IFN into the UBP43^{+} or UBP43^{-} recipient mice beginning at day 17 or day 28 post transplantation, respectively. At the starting date of injection, UBP43^{+} recipient mice showed ~50% EGFP+ cells in their peripheral blood, while UBP43^{-} recipients showed between 15-45% EGFP+ cells. Wild-type recipients rapidly succumb to the BCR-ABL induced myeloproliferative disease due to the aggressive nature of the disease and/or the lack of a sufficient response to the dosage of IFN used (Fig. 6A). In contrast, after 10 days of injections UBP43^{-} recipients showed ~50% decrease in the initial percentage of both donor cells and donor cells expressing BCR-ABL, whereas uninjected UBP43^{-} recipient mice over the same time period showed no such decrease (Fig. 6B).

Type I IFN signaling is critical for the resistance to CML development in mice receiving BCR-ABL transduced Ubp43 deficient bone marrow cells. To confirm the role of IFN in the resistance of CML development in Ubp43 deficient cells, we generated Ubp43 and IFN receptor R1 (Ifnar1) subunit double deficient bone marrow cells by crossing Ubp43 and Ifnar1 knockout mice. These double deficient mice showed normal general hematopoiesis. Bone marrow cells from wild type and double knockout mice were used to perform the BCR-ABL retroviral transduction and bone marrow transplantation assays. Loss of type I IFN signaling, through loss of the IFN-α/β receptor, resulted in a reversal of the original resistance to leukemia development observed for the recipients of Mig-p210 transduced Ubp43 deficient cells (Fig. 7A). The median survival times of mice with Mig-p210 transduced wild type and Ubp43$^{-/-}$ cells were 26 days and 33 days, respectively. The CML like pathological changes were similar in both types of mice except that substantially lower WBC counts were still observed in diseased recipients of Mig-p210-Ubp43$^{-/-}$/Ifnar1$^{-/-}$ bone marrow cells (Fig. 7B). These results demonstrate the crucial role of Type I IFN signaling in the resistance to CML development in Ubp43 deficient bone marrow cells.

Discussion

Using a well established mouse model for BCR-ABL induced CML-like myeloproliferative disease, we have shown that Ubp43 plays an important role in regulating the latency and severity of leukemia development. Furthermore, we have detected an increase in serum levels of endogenous Type I IFNs in leukemic mice. Importantly, we demonstrate that IFN signaling is critical for the inhibitory function of Ubp43 deficiency in leukemia development. This finding provides a novel molecular target to enhance innate immune responses of patients in treating various cancers that are responsive to IFN-α/β.

BCR-ABL induced CML is a hematopoietic stem cell disease. Before the ABL tyrosine kinase specific inhibitor imatinib became available, IFN-α had been the standard therapy choice for CML patients that were ineligible for bone marrow transplantation. Compared to IFN, imatinib is a very specific and effective drug, with fewer and less severe side effects. Most importantly, the initial response rate to imatinib is much higher than that of IFN.[49] However, concerns exist over the development of imatinib drug resistance, due to amplification and mutation of BCR-ABL, and the inability of the drug to eliminate BCR-ABL$^+$ stem cells from CML patients necessitating continued imatinib treatment even after achieving complete clinical response.[50;51] In contrast, although the molecular mechanism of the IFN clinical response is not fully understood, complete remission is achievable for a significant portion of IFN responsive patients even after stopping the administration of IFN.[52;53] Recently, interferon has been reported to have higher toxicity to the more primitive CML progenitors than imatinib.[54]

Therefore, IFN and specific reagents that can enhance the endogenous IFN response, via the inhibition of Ubp43 or other negative regulators of IFN signaling, can be useful drugs in combination with imatinib to treat BCR-ABL induced leukemia.

While the IFN signaling pathway is known in great detail,[5], the exact molecular mechanisms whereby IFN exerts its anti-tumor effects have eluded researchers. Indeed, studies have shown that IFN employs a multitude of pathways that are responsible for inhibiting translation, regulating cell cycle progression, and increasing apoptosis.[3;6] In vitro studies using immortalized MEFs demonstrated that IFN treatment failed to alter the expression or activation of BCR-ABL in the presence or the absence of Ubp43, suggesting that the major mechanism whereby IFN abrogates the oncogenic activity of BCR-ABL occurs further downstream. It was found that the BCR-ABL positive cells in the peripheral blood of mice carrying Mig-p210 transduced Ubp43^{-} bone marrow were more prone to apoptosis. The role of IFN and Ubp43 in inducing apoptosis of the leukemic cells is demonstrated both in shRNA studies of CML patient derived cell lines (Fig. 5), as well as direct injection of IFN into the recipient mice (Fig. 6). Therefore, the enhanced apoptosis due to the increased IFN production during CML development may be responsible for the relatively lower WBC count, the lesser degree of splenomegaly and hepatomegaly, and the significantly extended latency of disease in mice transplanted with Mig-p210 transduced Ubp43$^{-/-}$ bone marrow cells. The results presented in figures 4 and 6 also suggest that BCR-ABL expressing Ubp43 deficient hematopoietic cells are more sensitive to apoptotic stimulation than non-BCR-ABL expressing Ubp43 deficient cells. It has been reported that expression of BCR-ABL results in the increased tyrosine phosphorylation of multiple STAT family members

although the mechanism of this alteration is not clear.[55-58] This increased tyrosine phosphorylation of STATs may also enhance IFN induced JAK-STAT signaling via the complex formation of different STAT proteins and hence apoptosis. Ubp43 is a cysteine protease that catalyzes the removal of the ubiquitin-like modifier ISG15 from its conjugated targets.[18;59] Protein ISGylation is strongly induced by IFN stimulation,[60] as are all of the currently identified enzymes involved in ISG15 modification, including ISG15 E1 activating enzyme Ube1L, E2 conjugating enzyme UbcH8, E3 ligase Efp, and the deconjugating enzyme Ubp43.[15;16;25;44;61;62] This indicates that protein ISGylation may have important functions in innate immune responses. Ubp43 deficient cells have much higher levels of ISGylated proteins than wild type controls.[18] Furthermore, Ubp43 deficient cells have an enhanced and prolonged response to Type I IFN stimulation.[23] Here, we show that Ubp43 deficient cells are resistant to BCR-ABL induced leukemogenesis and this effect is mainly dependent on IFN signaling. We have recently demonstrated that the increased IFN sensitivity of Ubp43 deficient cells is mainly independent of its role in regulating the level of cellular protein ISGylation.[26;27] Therefore, the role of protein ISGylation in cancer development and the innate immune response remains to be identified.

Acknowledgments

We wish to thank members of Zhang lab for valuable discussions. This is manuscript 17892-MEM from The Scripps Research Institute.

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Figure Legends

Figure 1. Significant delay of CML development with Ubp43 deficient bone marrow cells in BCR-ABL retroviral transduction/transplantation assay. (A) Percentage of BCR/ABL expressing cells ($EGFP^+$) in peripheral blood of mice with Mig-p210 transduced and transplanted bone marrow cells. Each color represents the result from one individual mouse. BCR/ABL was expressed in either wild type $Ubp43^{+/+}$ or $Ubp43^{-/-}$ bone marrow cells (triangles). (B) Ubp43 is clearly detectable in the spleen of mice which develop the CML-like disease. RNA was prepared from the spleen of a mouse transplanted with MigR1 transduced $Ubp43^{+/+}$ bone marrow cells (control) and the spleen of a mouse with CML-like disease after transplantation with Mig-p210 infected wild type bone marrow cells (CML). Northern blot was performed with ^{32}P-labeled Ubp43 cDNA. Ethidium bromide stained 28S rRNA is shown for relative RNA loading. (C) Kaplan-Meier survival curve of mice transplanted with Mig-p210 or MigR1 transduced $Ubp43^{+/+}$, $Ubp43^{+/-}$, and $Ubp43^{-/-}$ bone marrow cells. The result is summarized from three separate sets of transplantation experiments. (D) Average WBC counts of MigR1 or Mig-p210 transduced $Ubp43^{+/+}$ and $Ubp43^{-/-}$ bone marrow cell recipients.

Figure 2. Pathological analysis of disease mice. (A) The average WBC counts and spleen weight of transplant recipientsat moribund. The error bars represent the standard deviation. (B) Histological analysis of spleens and livers of representative control and

experimental mice transplanted with Mig-p210 transduced Ubp43$^{+/+}$ and Ubp43$^{-/-}$ bone marrow cells. The tissue sections were stained by hematoxilin and eosin.

Figure 3. Elevated Type I IFN level is detected in the serum of mice with CML like disease. Serum was collected from control, MigR1 transplanted, and BCR-ABL induced CML mice.. The concentration of Type I IFN in these sera was measured as described in Materials and Methods. The relative concentrations of IFN in these sera are presented.

Figure 4. Increased apoptosis in Ubp43-deficient BCR/ABL^{+} cells in vivo. (A) Blood samples were collected from mice three to four weeks post transplantation with Mig-p210 transduced Ubp43$^{+/+}$ and Ubp43$^{-/-}$ bone marrow cells before disease emergence. Peripheral blood cells were stained with Annexin V and 7-AAD and the percentage of apoptotic cells measured by flow cytometry. (B) The average percentages of apoptotic peripheral blood EGFP^{+} cells (n = 3).

Figure 5. Increased apoptosis in Ubp43 shRNA expressing KT-1 cells treated with IFN. KT-1 cells stably transduced with the control or Ubp43 shRNA were treated with A) 1,000 units/ml or B) 100 units/ml hIFNα and the percentage increase in apoptotic cells (over untreated cells) was determined by Annexin V/7-AAD staining at various time points. The apoptotic percentage represents the sum of early (Annexin V positive) and late apoptotic (Annexin V/7-AAD double positive) percentages. The results are the mean $\pm$ SD of three separate experiments. A comparison of the apoptotic percentage

at 72h in control shRNA expressing cells to that of UBP43 shRNA expressing cells, yields *P* values of A) 0.05 and B) 0.02. C) KT-1 cells expressing the various shRNAs were treated with 1,000 units/ml hIFNα. Western blots of the lysates at various time points were probed with anti-cytochrome c, anti-TRAIL, or anti-caspase 3 antibodies. Ponceau stains of the blots are shown as protein loading controls.

Figure 6. IFN injection into Ubp43-deficient BCR/ABL$^+$ recipient mice reduces the leukemic cell percentage. A) Recipients of BCR-ABL transduced UBP43$^+$ (n = 2) or UBP43$^-$ (n = 5) were injected subcutaneously with 18,000 units/day mIFNβ beginning at day 17 or day 28, respectively. The graph is a plot of the percentage change over time in total donor cells (CD45.2 positive) and BCR-ABL positive donor cells (EGFP+) from the start date of the injection (day 0). UBP43$^+$ recipient mice rapidly succumbed to BCR-ABL induced leukemia whereas UBP43$^-$ recipients showed a decrease in the donor cell numbers up to a maximum of ~50% at day 10. (B) Uninjected UBP43$^-$ recipients (n = 4) showed no decrease in the donor cell percentage over the same time period.

Figure 7. IFNα/β signaling plays a critical role in resistance to leukemia development in Ubp43 deficient cells. (A) Kaplan-Meier survival curve of mice transplanted with MigR1 or Mig-p210 transduced wild type bone marrow cells (WT) or Ubp43 and IFNα/β receptor subunit R1 (Ifnar1) double deficient bone marrow cells (DK). (B) WBC counts and spleen weight of moribund mice.

Supplemental Figure 1. Ubp43 deficient bone marrow cells are suitable for bone marrow cell retroviral infection and transplantation. The mean and the standard deviation of the percentage of WBCs expressing retrovirally transduced $EGFP^{+}$ post bone marrow transplantation (BMT).

Supplemental Figure 2. The expression and phosphorylation of BCR-ABL are not affected by the presence of Ubp43 or IFN-β. Protein extracts were prepared from parental $Ubp43^{+/+}$, $Ubp43^{-/-}$, and Ubp43 reconstituted $Ubp43^{-/-}$ (Ubp43reconst) MEFs and BCR-ABL expressing MEFs. (A) Ten μg of protein from each sample was used to western blot sequentially with α-ABL and α-tubulin antibodies. (B) The same protein samples were used to western blot with the antibody against phosphorylated tyrosine. BCR-ABL transduced lines showed a high molecular weight band (indicated by the arrow, > 175 kDa) corresponding to phosphorylated BCR-ABL, which is reduced upon STI571 treatment (data not shown). A constitutively phosphorylated protein (indicated by *) served as a loading control.

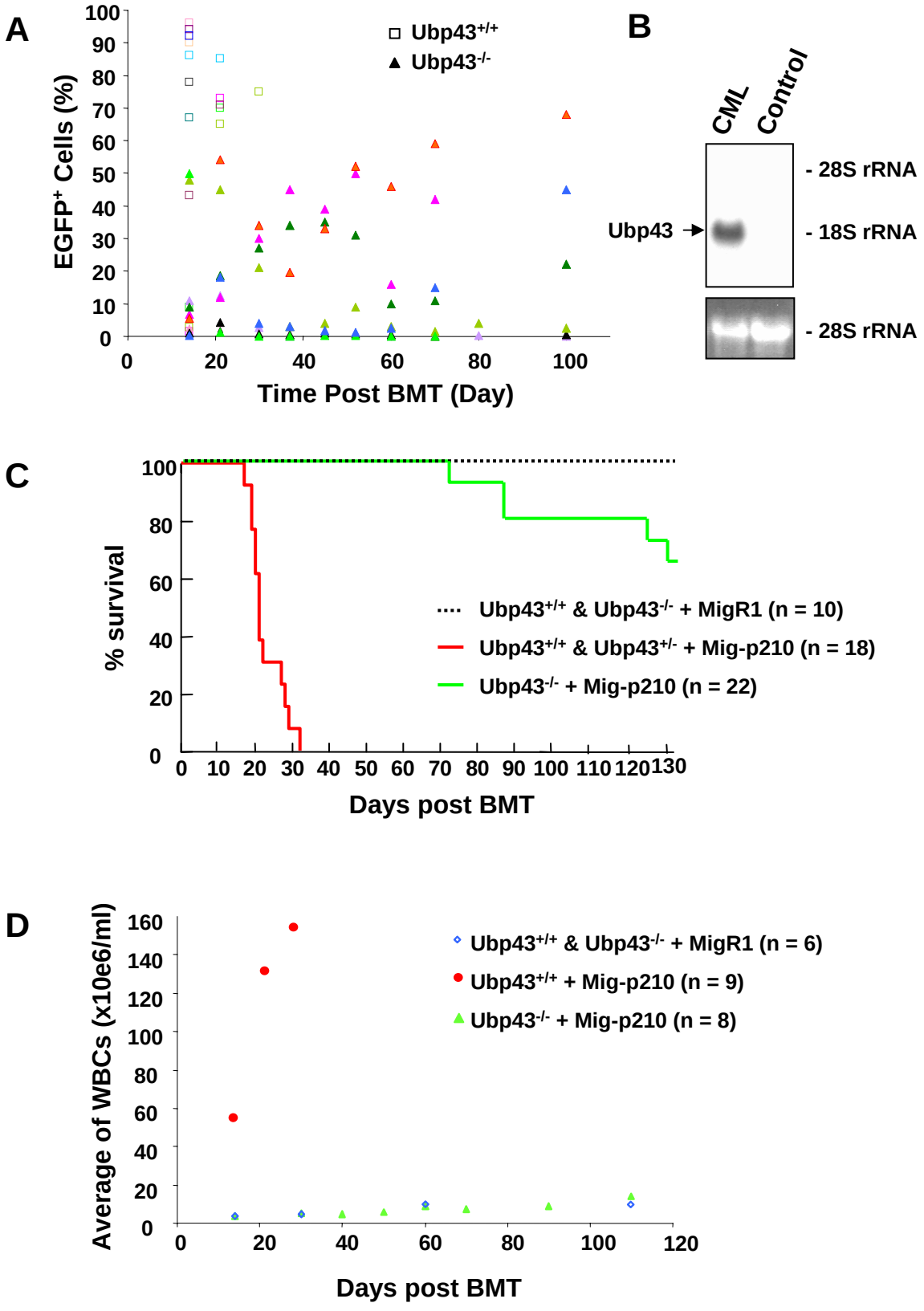


Figure 1, Yan et al.

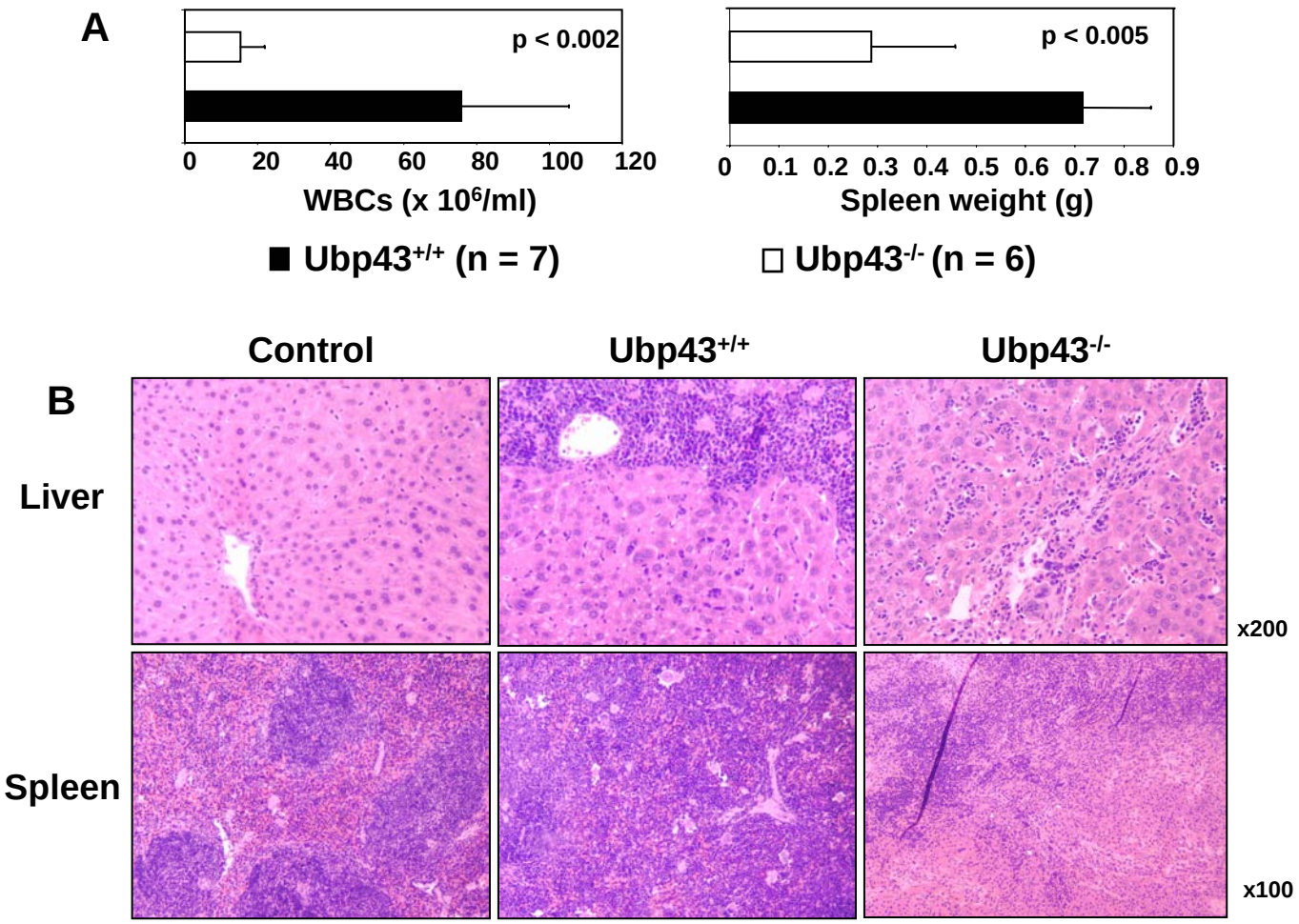
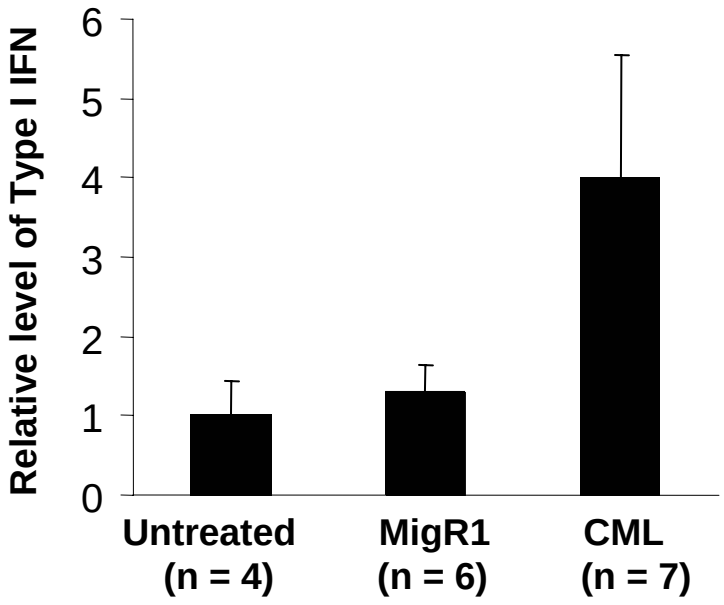


Figure 2, Yan et al.



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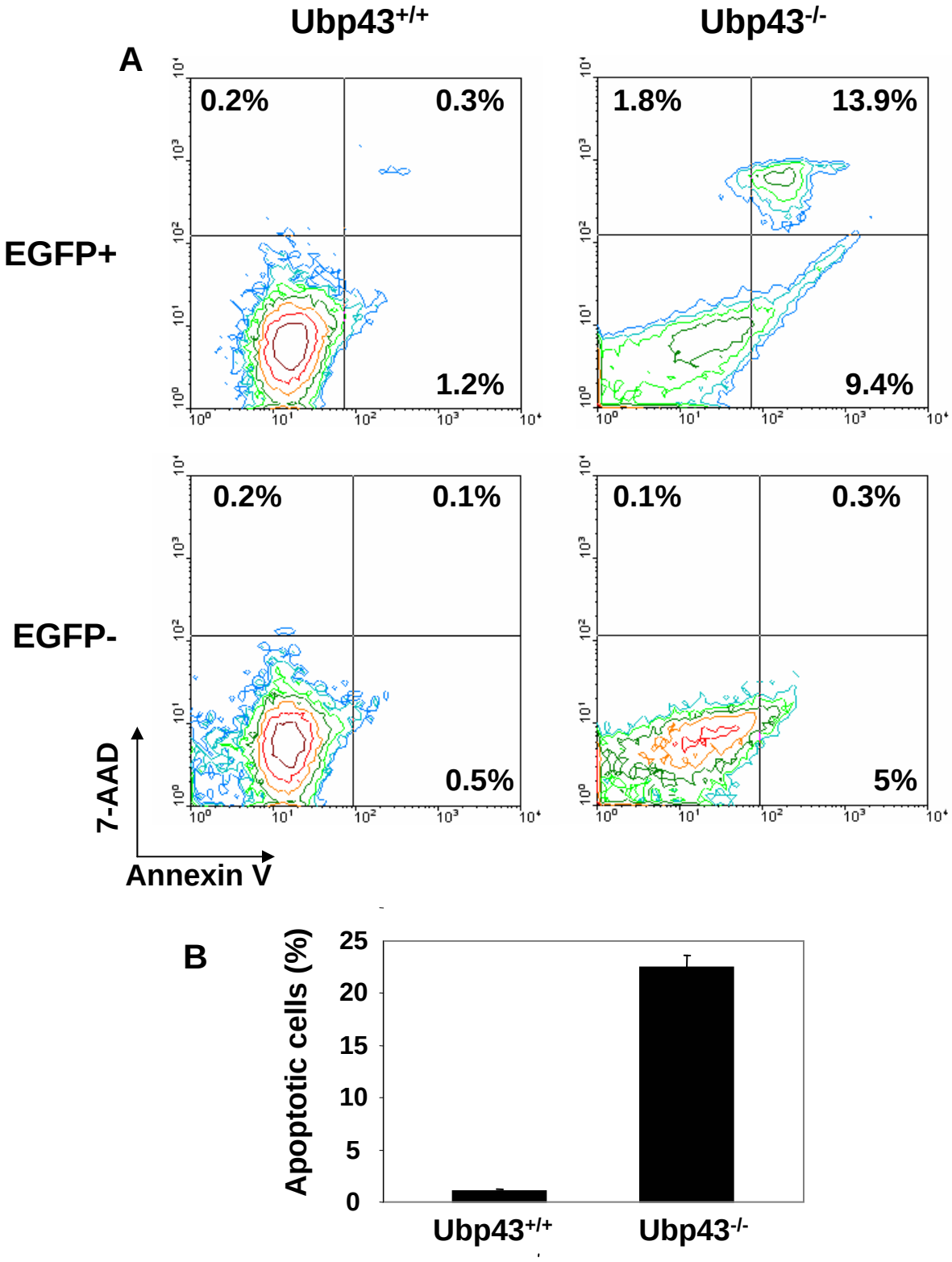

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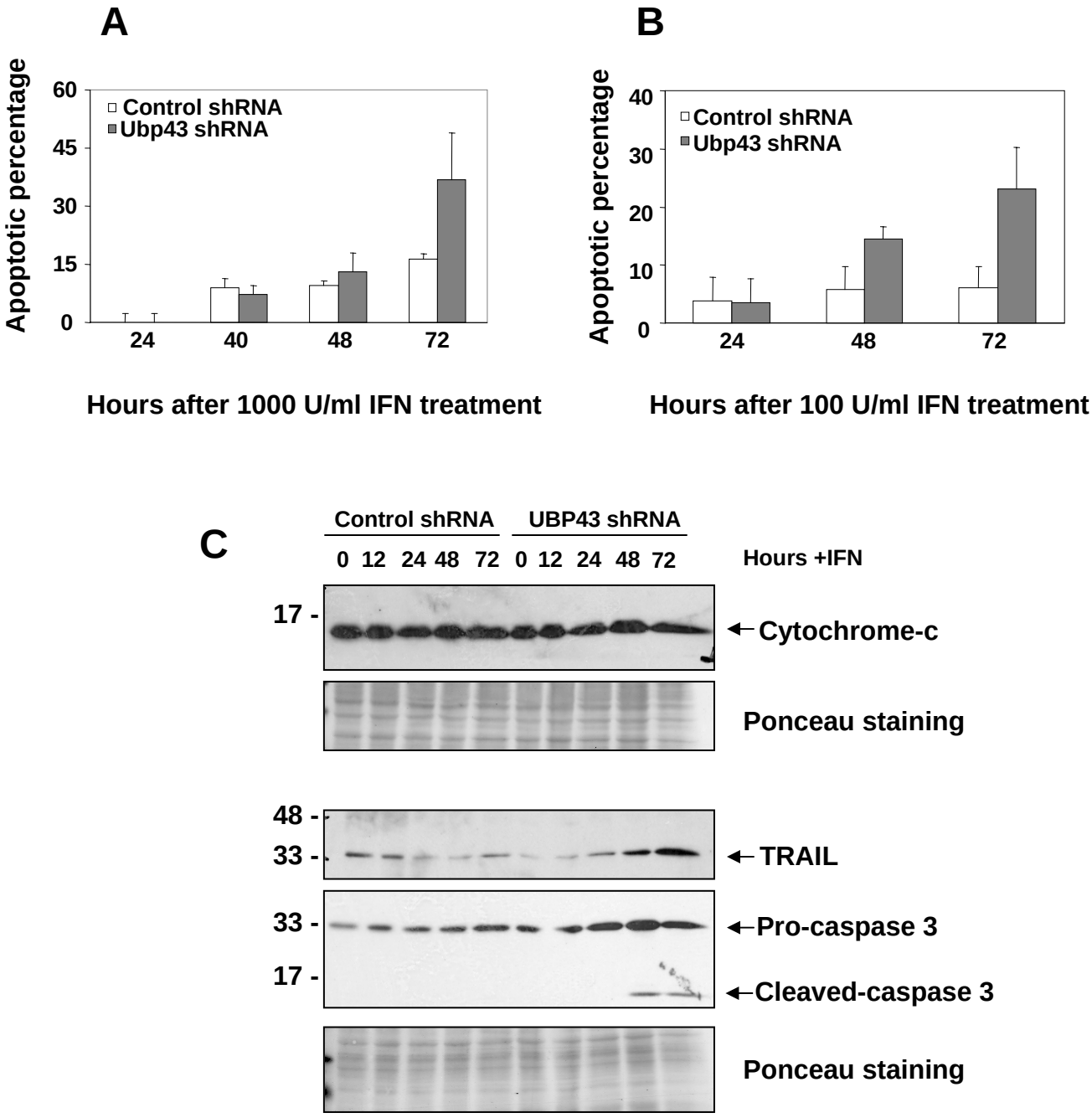


Figure 5, Yan et al.

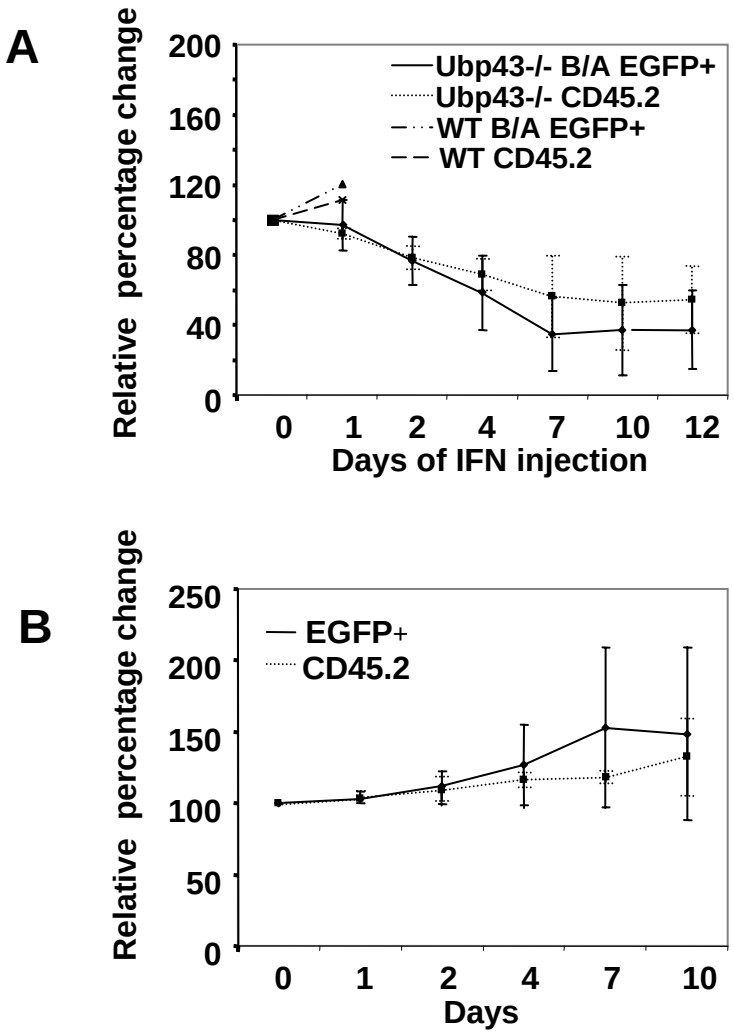


Figure 6, Yan et al.

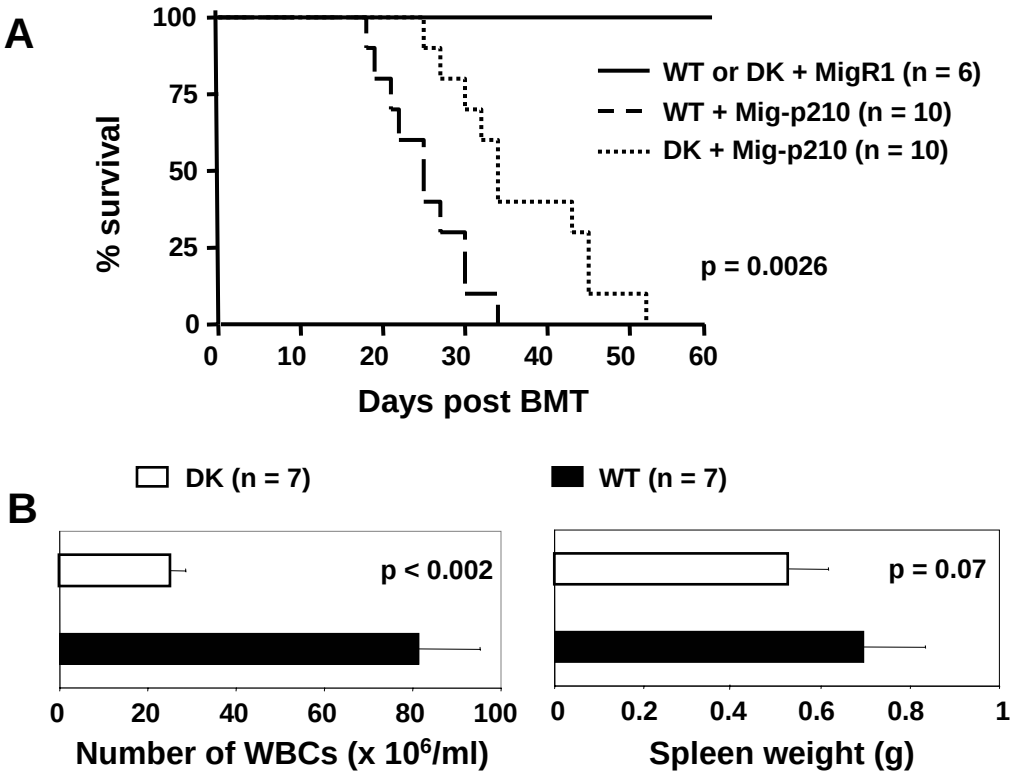
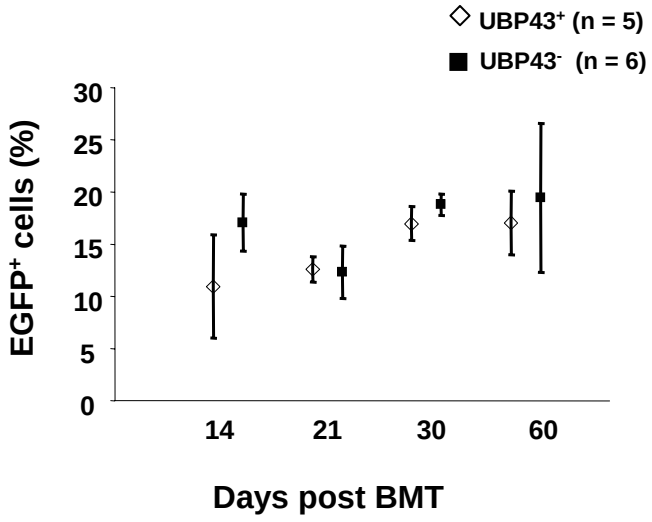
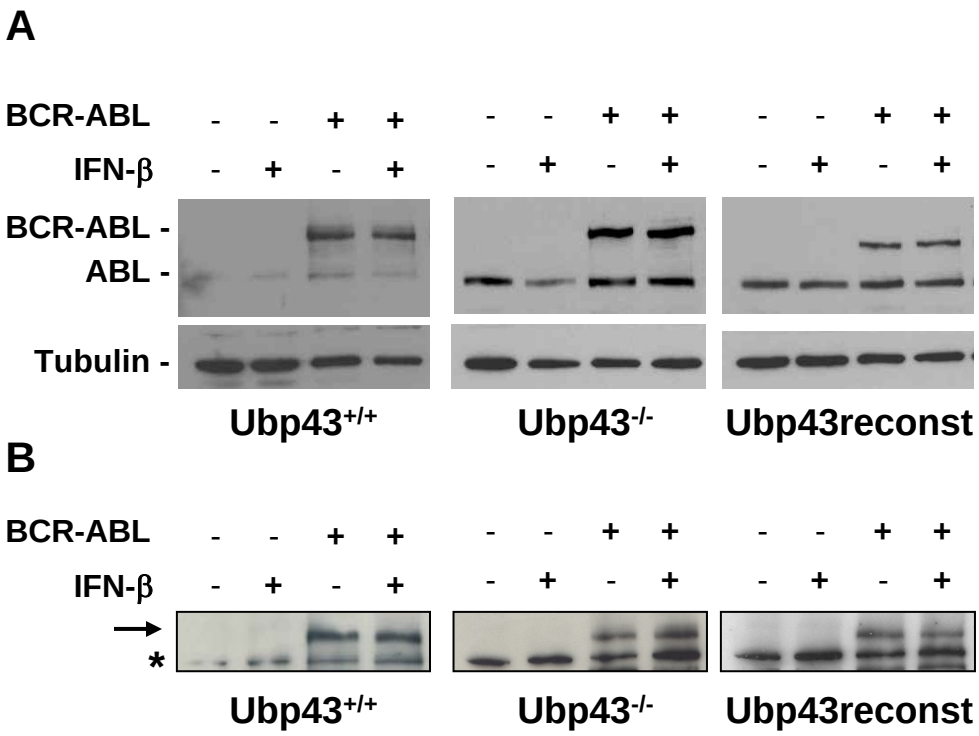


Figure 7, Yan et al.



Supplemental Figure 1, Yan et al.



Supplemental Figure 2, Yan et al.